



PCT/GB 2004 / 0 0 5 0 0 6



INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

REC'D 17 JAN 2005

WIPO

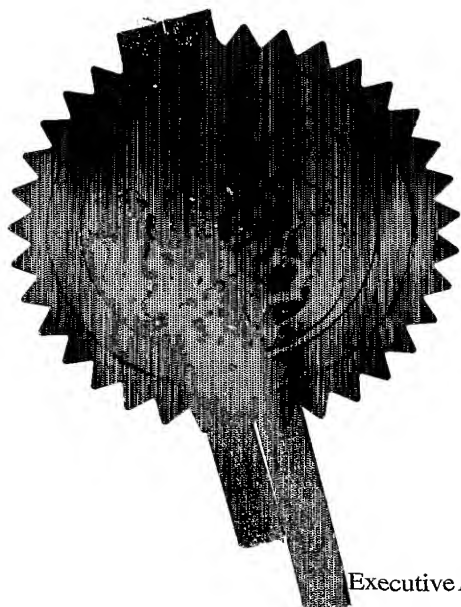
PCT

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.



Signed

L. Mahoney

Dated 4 January 2005

**PRIORITY
DOCUMENT**
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

Executive Agency of the Department of Trade and Industry

Patents Form 1/77

Patents Act 1977
(Rule 16)

THE PATENT OFFICE
SW
26 NOV 2003
RECEIVED BY FAX



177

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
South Wales
NP10 8QQ

1. Your reference

P34234-/CMU/MCM

26NOV03 0955185-1 002884

2. Patent application number

(The Patent Office will fill in this)

0327493.3

P01/7700 0.00-0327493.3

26 NOV 2003

3. Full name, address and postcode of the or of each applicant (*underline all surnames*)

The Queen's University of Belfast
University Road
Belfast
BT7 1NN

Patents ADP number (*if you know it*)

889659006

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

4. Title of the invention

"Treatment Medicament"

5. Name of your agent (*if you have one*)

Murgitroyd & Company

"Address for service" in the United Kingdom
to which all correspondence should be sent
(including the postcode)

Scotland House
165-169 Scotland Street
Glasgow
G5 8PL

Patents ADP number (*if you know it*)

1198013 S

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (*if you know it*) the or each application number

Country

Priority application number
(*if you know it*)Date of filing
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing
(day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (*Answer 'Yes' if*

Yes

- a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

Patents Form 1/77

0087107 26-Nov-03 04:38

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form

Description

59 /

Claim(s)

5 /

Abstract

-

Drawing(s)

20 /

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (*Patents Form 7/77*)

Request for preliminary examination and search (*Patents Form 9/77*)

Request for substantive examination (*Patents Form 10/77*)

Any other documents
(*please specify*)

11.

I/We request the grant of a patent on the basis of this application.

Signature

Murgitroyd & Company

Date

26 November 2003

12. Name and daytime telephone number of person to contact in the United Kingdom

MALCOLM MAIN

0141 307 8400

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

- If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.
- Write your answers in capital letters using black ink or you may type them.
- If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.
- If you have answered 'Yes' Patents Form 7/77 will need to be filed.
- Once you have filled in the form you must remember to sign and date it.
- For details of the fee and ways to pay please contact the Patent Office.

Patents Form 1/77

0087107*26-Nov-03 04:38

DUPLICATE

1

1 "Treatment Medicament"

2
3 Field of the Invention

4
5 This application relates to a medicament and its use
6 in methods of treatment. In particular, it relates
7 to the treatment of cancer with a death receptor
8 ligand, e.g. a FAS (CD95 or TNF receptor 2) receptor
9 ligand, and a chemotherapeutic agent.

10
11 Background to the Invention

12
13 Breast, oesophageal, colorectal, all forms of GI
14 cancer and head and neck cancers are highly
15 malignant with overall 5-year survival rates of less
16 than 50%. The clinical outcome of these patients is
17 predetermined by the presence of widely disseminated
18 tumour cells termed micrometastases with potential
19 for metastatic growth, prior to clinical
20 presentation. Approximately 50% of oesophageal
21 cancer patients are selected for surgical therapy

1 with 30% 5-year survival for this patient sub-group.
2 Randomised clinical trials of neoadjuvant 5FU-based
3 chemotherapy combined with fractionated radiotherapy
4 have demonstrated improvements in survival of 10-
5 20%, although the overall 5-year outcome for the
6 treated groups remains at 30-35%. Those patients
7 who demonstrate complete pathological response in
8 their primary tumours as a result of neoadjuvant
9 treatment have a five-year survival of 80%.
10 Conversely, those patients who do not respond to
11 5FU-based chemotherapy are denied the opportunity
12 for earlier treatment by surgery or a different
13 neoadjuvant chemotherapeutic based regimen. Thus,
14 there is an urgent need for improved therapeutic
15 strategies.
16
17 5-FU¹ is widely used in the treatment of a range of
18 cancers including colorectal, breast and cancers of
19 the aerodigestive tract. The mechanism of
20 cytotoxicity of 5-FU has been ascribed to the
21 misincorporation of fluoronucleotides into RNA and
22 DNA and to the inhibition of the nucleotide
23 synthetic enzyme thymidylate synthase (TS) (1). TS
24 catalyses the conversion of deoxyuridine
25 monophosphate (dUMP) to deoxythymidine monophosphate
26 (dTTP) with 5,10-methylene tetrahydrofolate (CH₂THF)
27 as the methyl donor. This reaction provides the sole
28 intracellular source of thymidylate, which is
29 essential for DNA synthesis and repair. The 5-FU
30 metabolite fluorodeoxyuridine monophosphate (FdUMP)
31 forms a stable complex with TS and CH₂THF resulting
32 in enzyme inhibition (1). Recently, more specific

1 folate-based inhibitors of TS have been developed
2 such as RTX and MTA, which form a stable complex
3 with TS and dUMP that inhibits binding of CH₂THF to
4 the enzyme (2, 3). TS inhibition causes nucleotide
5 pool imbalances that result in S phase cell cycle
6 arrest and apoptosis (4-6).

7

8

9 Summary of the Invention

10

11 As described herein, the present inventors have
12 surprisingly shown that by combining treatment using
13 a death receptor ligand, such as an anti FAS
14 antibody, with a chemotherapeutic agent such as 5-FU
15 or an antifolate drug, such as raltitrexed (RTX) or
16 pemetrexed (MTA, Alimta), a synergistic effect is
17 achieved in the killing of cancer cells.

18

19 Accordingly, in a first aspect, the present
20 invention provides a method of killing cancer cells
21 comprising administration of a therapeutically
22 effective amount of a) a specific binding member
23 which binds to a cell death receptor or a nucleic
24 acid encoding said binding member and (b) a
25 chemotherapeutic agent.

26

27 In a second aspect, the present invention provides a
28 method of treating cancer comprising administration
29 of a therapeutically effective amount of a) a
30 specific binding member which binds to a cell death
31 receptor or a nucleic acid encoding said binding

1 member and (b) a chemotherapeutic agent to a mammal
2 in need thereof.

3

4 The specific binding member and the chemotherapeutic
5 agent may be administered simultaneously,
6 sequentially or simultaneously. In preferred
7 embodiments of the invention, the chemotherapeutic
8 agent is administered prior to the specific binding
9 member.

10

11 In a third aspect, there is provided the use of (a)
12 a specific binding member which binds to a cell
13 death receptor or a nucleic acid encoding said
14 binding member and (b) a chemotherapeutic agent in
15 the preparation of a medicament for treating cancer.
16

17 In a fourth aspect, there is provided a product
18 comprising a) a specific binding member which binds
19 to a cell death receptor or a nucleic acid encoding
20 said binding member and (b) a chemotherapeutic agent
21 as a combined preparation for the simultaneous,
22 separate or sequential use in the treatment of
23 cancer.

24

25 According to a fifth aspect, there is provided a
26 pharmaceutical composition for the treatment of
27 cancer, wherein the composition comprises a) a
28 specific binding member which binds to a cell death
29 receptor or a nucleic acid encoding said binding
30 member and (b) a chemotherapeutic agent and (c) a
31 pharmaceutically acceptable excipient, diluent or

1 carrier.

2

3 In a sixth aspect, there is provided a kit for the
4 treatment of cancer, said kit comprising a) a
5 specific binding member which binds to a cell death
6 receptor or a nucleic acid encoding said binding
7 member and (b) a chemotherapeutic agent and (c)
8 instructions for the administration of (a) and (b)
9 separately, sequentially or simultaneously.

10

11 The invention may be used to treat any cancer. In
12 preferred embodiments of the invention, the cancer
13 is one or more of colorectal, breast , ovarian,
14 cervical, gastric, lung, liver, skin and myeloid
15 (e.g. bone marrow) cancer.

16

17 In preferred embodiments of the invention, the
18 binding member is an antibody or a fragment thereof.
19 In particularly preferred embodiments, the binding
20 member is the FAS antibody CH11 (Yonehara, S.,
21 Ishii, A. and Yonehara, M. (1989) J. Exp. Med. 169,
22 1747-1756) (available commercially e.g. from Upstate
23 Biotechnology, Lake Placid, NY).

24

25 The binding member may bind to any death receptor.
26 Death receptors include, Fas, TNFR, DR-3, DR-4 and
27 DR-5. In preferred embodiments of the invention, the
28 death receptor is FAS.

29

30 In preferred embodiments, the binding member
31 comprises at least one human constant region.

32

1 Any suitable chemotherapeutic agent may be used in
2 the present invention. In preferred embodiments, the
3 agent is doxorubicin, oxaliplatin, taxol, tomudex
4 (TDX), 5-Fluorouracil (5-FU), Irinotecan (CPT11) or
5 an antifolate e.g. MTA or RTX. In one preferred
6 embodiment, the agent is tomudex, 5-Fluorouracil, an
7 antifolate (for example RTX or MTA), or a
8 combination thereof. In a particularly preferred
9 embodiment, the agent is 5-FU or an antifolate. In
10 another preferred embodiment, the agent is an
11 antifolate. In a particularly preferred embodiment
12 the agent is MTA.

13
14 The invention also provides a method of treating
15 tumour cells, the method including the steps of
16 administering a compound capable of triggering or
17 binding a death receptor, e.g. a binding member and
18 administering a chemotherapeutic agent.

19
20 The concentrations of binding members and
21 chemotherapeutic agents used are preferably
22 sufficient to provide a synergistic effect.
23 Synergism is preferably defined as an RI of greater
24 than unity using the method of Kern as modified by
25 Romaneli (13, 14). The RI may be calculated as the
26 ratio of expected cell survival (S_{exp} , defined as the
27 product of the survival observed with drug A alone
28 and the survival observed with drug B alone) to the
29 observed cell survival (S_{obs}) for the combination of
30 A and B ($RI = S_{exp}/S_{obs}$). Synergism may then be defined
31 as an RI of greater than unity.

32

1 In preferred embodiments of the invention, said
2 specific binding member and chemotherapeutic agent
3 are provided in concentrations sufficient to produce
4 an RI of greater than 1.5, more preferably greater
5 than 2.0, most preferably greater than 2.25.

6
7 The combined medicament thus preferably produces a
8 synergistic effect when used to treat tumour cells.

9
10 A seventh aspect of the present invention therefore
11 provides a medicament for use in treating tumour
12 cells, the medicament comprising at least one
13 antibody directed at FAS receptor and at least one
14 cancer chemotherapeutic agent.

15
16 The invention also provides in a eighth aspect a
17 method of treating tumour cells, the method
18 including the steps of administering a compound
19 capable of triggering or binding a death receptor
20 and administering simultaneously, sequentially or
21 separately a chemotherapeutic agent.

22
23 In an ninth aspect, the invention provides the use
24 of an antibody directed at FAS receptor in
25 combination with a cancer chemotherapeutic agent in
26 the preparation of a medicament for treatment of
27 tumour cells.

28
29 In a particular aspect, the application relates to
30 the use of an antibody or a fas ligand directed at a
31 death receptor e.g. the FAS receptor (CD95/TNF
32 receptor 2) to synergise with cancer

1 chemotherapeutic agents, e.g. 5-FU or an antifolate,
2 for example RTX or MTA, to enhance therapy and
3 enhance the removal or regression of tumour cells.
4

5 This application is relevant for, but is not limited
6 to, breast cancer, oesophageal cancer, colorectal
7 cancer, all forms of GI cancer and head and neck
8 cancers and may also be used to target other cells
9 via death receptors.
10

11 Preferred features of each aspect of the invention
12 are as for each of the other aspects mutatis
13 mutandis.
14

15 Detailed Description

16 Binding members

17
18
19 In the context of the present invention, a "binding
20 member" is a molecule which has binding specificity
21 for another molecule, in particular a receptor, in
22 particular a death receptor. The binding member may
23 be a member of a pair of specific binding members.
24 The members of a binding pair may be naturally
25 derived or wholly or partially synthetically
26 produced. One member of the pair of molecules may
27 have an area on its surface, which may be a
28 protrusion or a cavity, which specifically binds to
29 and is therefore complementary to a particular
30 spatial and polar organisation of the other member
31 of the pair of molecules. Thus, the members of the
32 pair have the property of binding specifically to

1 each other. Examples of types of binding pairs are
2 antigen-antibody, biotin-avidin, hormone-hormone
3 receptor, receptor-ligand, enzyme-substrate. A
4 binding member of the invention and for use in the
5 invention may be any moiety, for example an antibody
6 or ligand, which can bind to a death receptor.

7

8 Antibodies

9

10 An "antibody" is an immunoglobulin, whether natural
11 or partly or wholly synthetically produced. The
12 term also covers any polypeptide, protein or peptide
13 having a binding domain which is, or is homologous
14 to, an antibody binding domain. These can be
15 derived from natural sources, or they may be partly
16 or wholly synthetically produced. Examples of
17 antibodies are the immunoglobulin isotypes and their
18 isotypic subclasses and fragments which comprise an
19 antigen binding domain such as Fab, scFv, Fv, dAb,
20 Fd; and diabodies.

21

22 The binding member of the invention may be an
23 antibody such as a monoclonal or polyclonal
24 antibody, or a fragment thereof. The constant region
25 of the antibody may be of any class including, but
26 not limited to, human classes IgG, IgA, IgM, IgD and
27 IgE. The antibody may belong to any sub class e.g.
28 IgG1, IgG2, IgG3 and IgG4. IgG1 is preferred.

29

30 As antibodies can be modified in a number of ways,
31 the term "antibody" should be construed as covering
32 any binding member or substance having a binding

1 domain with the required specificity. Thus, this
2 term covers antibody fragments, derivatives,
3 functional equivalents and homologues of antibodies,
4 including any polypeptide comprising an
5 immunoglobulin binding domain, whether natural or
6 wholly or partially synthetic. Chimeric molecules
7 comprising an immunoglobulin binding domain, or
8 equivalent, fused to another polypeptide are
9 therefore included. Cloning and expression of
10 chimeric antibodies are described in EP-A-0120694
11 and EP-A-0125023.

12
13 It has been shown that fragments of a whole antibody
14 can perform the function of binding antigens.
15 Examples of such binding fragments are (i) the Fab
16 fragment consisting of VL, VH, CL and CH1 domains;
17 (ii) the Fd fragment consisting of the VH and CH1
18 domains; (iii) the Fv fragment consisting of the VL
19 and VH domains of a single antibody; (iv) the dAb
20 fragment (Ward, E.S. et al., Nature 341:544-546
21 (1989)) which consists of a VH domain; (v) isolated
22 CDR regions; (vi) F(ab')₂ fragments, a bivalent
23 fragment comprising two linked Fab fragments (vii)
24 single chain Fv molecules (scFv), wherein a VH
25 domain and a VL domain are linked by a peptide
26 linker which allows the two domains to associate to
27 form an antigen binding site (Bird et al., Science
28 242:423-426 (1988); Huston et al., PNAS USA 85:5879-
29 5883 (1988)); (viii) bispecific single chain Fv
30 dimers (PCT/US92/09965) and (ix) "diabodies",
31 multivalent or multispecific fragments constructed

1 by gene fusion (WO94/13804; P. Hollinger et al.,
2 Proc. Natl. Acad. Sci. USA 90:6444-6448 (1993)).

3
4 A fragment of an antibody or of a polypeptide for
5 use in the present invention generally means a
6 stretch of amino acid residues of at least 5 to 7
7 contiguous amino acids, often at least about 7 to 9
8 contiguous amino acids, typically at least about 9
9 to 13 contiguous amino acids, more preferably at
10 least about 20 to 30 or more contiguous amino acids
11 and most preferably at least about 30 to 40 or more
12 consecutive amino acids.

13
14 A "derivative" of such an antibody or polypeptide,
15 or of a fragment antibody means an antibody or
16 polypeptide modified by varying the amino acid
17 sequence of the protein, e.g. by manipulation of the
18 nucleic acid encoding the protein or by altering the
19 protein itself. Such derivatives of the natural
20 amino acid sequence may involve insertion, addition,
21 deletion and/or substitution of one or more amino
22 acids, preferably while providing a peptide having
23 death receptor, e.g. FAS neutralisation and/or
24 binding activity. Preferably such derivatives
25 involve the insertion, addition, deletion and/or
26 substitution of 25 or fewer amino acids, more
27 preferably of 15 or fewer, even more preferably of
28 10 or fewer, more preferably still of 4 or fewer and
29 most preferably of 1 or 2 amino acids only.

30
31 The term "antibody" includes antibodies which have
32 been "humanised". Methods for making humanised

1 antibodies are known in the art. Methods are
2 described, for example, in Winter, U.S. Patent No.
3 5,225,539. A humanised antibody may be a modified
4 antibody having the hypervariable region of a
5 monoclonal antibody and the constant region of a
6 human antibody. Thus the binding member may
7 comprise a human constant region.

8
9 The variable region other than the hypervariable
10 region may also be derived from the variable region
11 of a human antibody and/or may also be derived from
12 a monoclonal antibody. In such case, the entire
13 variable region may be derived from murine
14 monoclonal antibody and the antibody is said to be
15 chimerised. Methods for making chimerised
16 antibodies are known in the art. Such methods
17 include, for example, those described in U.S.
18 patents by Boss (Celltech) and by Cabilly
19 (Genentech). See U.S. Patent Nos. 4,816,397 and
20 4,816,567, respectively.

21
22 It is possible to take monoclonal and other
23 antibodies and use techniques of recombinant DNA
24 technology to produce other antibodies or chimeric
25 molecules which retain the specificity of the
26 original antibody. Such techniques may involve
27 introducing DNA encoding the immunoglobulin variable
28 region, or the complementary determining regions
29 (CDRs), of an antibody to the constant regions, or
30 constant regions plus framework regions, of a
31 different immunoglobulin. See, for instance, EP-A-
32 184187, GB 2188638A or EP-A-239400. A hybridoma or

1 other cell producing an antibody may be subject to
2 genetic mutation or other changes, which may or may
3 not alter the binding specificity of antibodies
4 produced.

5
6 A typical antibody for use in the present invention
7 is a humanised equivalent of CH11 or any chimerised
8 equivalent of an antibody that can bind to the FAS
9 receptor and any alternative antibodies directed at
10 the FAS receptor that have been chimerised and can
11 be use in the treatment of humans. Furthermore, the
12 typical antibody is any antibody that can cross-
13 react with the extracellular portion of the FAS
14 receptor and either bind with high affinity to the
15 FAS receptor, be internalised with the FAS receptor
16 or trigger signalling through the FAS receptor.

17

18 Production of Binding Members

19

20 The binding members for use in the present invention
21 may be generated wholly or partly by chemical
22 synthesis. The binding members can be readily
23 prepared according to well-established, standard
24 liquid or, preferably, solid-phase peptide synthesis
25 methods, general descriptions of which are broadly
26 available (see, for example, in J.M. Stewart and
27 J.D. Young, Solid Phase Peptide Synthesis, 2nd
28 edition, Pierce Chemical Company, Rockford, Illinois
29 (1984), in M. Bodanzsky and A. Bodanzsky, The
30 Practice of Peptide Synthesis, Springer Verlag, New
31 York (1984); and Applied Biosystems 430A Users
32 Manual, ABI Inc., Foster City, California), or they

14

1 may be prepared in solution, by the liquid phase
2 method or by any combination of solid-phase, liquid
3 phase and solution chemistry, e.g. by first
4 completing the respective peptide portion and then,
5 if desired and appropriate, after removal of any
6 protecting groups being present, by introduction of
7 the residue X by reaction of the respective carbonic
8 or sulfonic acid or a reactive derivative thereof.

9
10 Another convenient way of producing a binding member
11 suitable for use in the present invention is to
12 express nucleic acid encoding it, by use of nucleic
13 acid in an expression system. Thus the present
14 invention further provides the use of (a) nucleic
15 acid encoding a specific binding member which binds
16 to a cell death receptor and (b) a chemotherapeutic
17 agent in the preparation of a medicament for
18 treating cancer.

19
20 Nucleic acid for use in accordance with the present
21 invention may comprise DNA or RNA and may be wholly
22 or partially synthetic. In a preferred aspect,
23 nucleic acid for use in the invention codes for a
24 binding member of the invention as defined above.
25 The skilled person will be able to determine
26 substitutions, deletions and/or additions to such
27 nucleic acids which will still provide a binding
28 member suitable for use in the present invention.

29
30 Nucleic acid sequences encoding a binding member for
31 use with the present invention can be readily
32 prepared by the skilled person using the information

1 and references contained herein and techniques known
2 in the art (for example, see Sambrook, Fritsch and
3 Maniatis, "Molecular Cloning", A Laboratory Manual,
4 Cold Spring Harbor Laboratory Press, 1989, and
5 Ausubel et al, Short Protocols in Molecular Biology,
6 John Wiley and Sons, 1992), given the nucleic acid
7 sequences and clones available. These techniques
8 include (i) the use of the polymerase chain reaction
9 (PCR) to amplify samples of such nucleic acid, e.g.
10 from genomic sources, (ii) chemical synthesis, or
11 (iii) preparing cDNA sequences. DNA encoding
12 antibody fragments may be generated and used in any
13 suitable way known to those of skill in the art,
14 including by taking encoding DNA, identifying
15 suitable restriction enzyme recognition sites either
16 side of the portion to be expressed, and cutting out
17 said portion from the DNA. The portion may then be
18 operably linked to a suitable promoter in a standard
19 commercially available expression system. Another
20 recombinant approach is to amplify the relevant
21 portion of the DNA with suitable PCR primers.
22 Modifications to the sequences can be made, e.g.
23 using site directed mutagenesis, to lead to the
24 expression of modified peptide or to take account of
25 codon preferences in the host cells used to express
26 the nucleic acid.

27

28 The nucleic acid may be comprised as construct(s) in
29 the form of a plasmid, vector, transcription or
30 expression cassette which comprises at least one
31 nucleic acid as described above. The construct may
32 be comprised within a recombinant host cell which

1 comprises one or more constructs as above.
2 Expression may conveniently be achieved by culturing
3 under appropriate conditions recombinant host cells
4 containing the nucleic acid. Following production
5 by expression a specific binding member may be
6 isolated and/or purified using any suitable
7 technique, then used as appropriate.
8

9 Binding members-encoding nucleic acid molecules and
10 vectors for use in accordance with the present
11 invention may be provided isolated and/or purified,
12 e.g. from their natural environment, in
13 substantially pure or homogeneous form, or, in the
14 case of nucleic acid, free or substantially free of
15 nucleic acid or genes origin other than the sequence
16 encoding a polypeptide with the required function.
17

18 Systems for cloning and expression of a polypeptide
19 in a variety of different host cells are well known.
20 Suitable host cells include bacteria, mammalian
21 cells, yeast and baculovirus systems. Mammalian
22 cell lines available in the art for expression of a
23 heterologous polypeptide include Chinese hamster
24 ovary cells, HeLa cells, baby hamster kidney cells,
25 NSO mouse melanoma cells and many others. A common,
26 preferred bacterial host is *E. coli*.
27

28 The expression of antibodies and antibody fragments
29 in prokaryotic cells such as *E. coli* is well
30 established in the art. For a review, see for
31 example Plückthun, *Bio/Technology* 9:545-551 (1991).
32 Expression in eukaryotic cells in culture is also

1 available to those skilled in the art as an option
2 for production of a binding member, see for recent
3 review, for example Reff, Curr. Opinion Biotech.
4 4:573-576 (1993); Trill et al., Curr. Opinion
5 Biotech. 6:553-560 (1995).

6
7 Suitable vectors can be chosen or constructed,
8 containing appropriate regulatory sequences,
9 including promoter sequences, terminator sequences,
10 polyadenylation sequences, enhancer sequences,
11 marker genes and other sequences as appropriate.
12 Vectors may be plasmids, viral e.g. 'phage, or
13 phagemid, as appropriate. For further details see,
14 for example, Sambrook et al., Molecular Cloning: A
15 Laboratory Manual: 2nd Edition, Cold Spring Harbor
16 Laboratory Press (1989). Many known techniques and
17 protocols for manipulation of nucleic acid, for
18 example in preparation of nucleic acid constructs,
19 mutagenesis, sequencing, introduction of DNA into
20 cells and gene expression, and analysis of proteins,
21 are described in detail in Ausubel et al. eds.,
22 Short Protocols in Molecular Biology, 2nd Edition,
23 John Wiley & Sons (1992).

24
25 The nucleic acid may be introduced into a host cell
26 by any suitable means. The introduction may employ
27 any available technique. For eukaryotic cells,
28 suitable techniques may include calcium phosphate
29 transfection, DEAE-Dextran, electroporation,
30 liposome-mediated transfection and transduction
31 using retrovirus or other virus, e.g. vaccinia or,
32 for insect cells, baculovirus. For bacterial cells,

18

1 suitable techniques may include calcium chloride
2 transformation, electroporation and transfection
3 using bacteriophage.

4

5 Marker genes such as antibiotic resistance or
6 sensitivity genes may be used in identifying clones
7 containing nucleic acid of interest, as is well
8 known in the art.

9

10 The introduction may be followed by causing or
11 allowing expression from the nucleic acid, e.g. by
12 culturing host cells under conditions for expression
13 of the gene.

14

15 The nucleic acid may be integrated into the genome
16 (e.g. chromosome) of the host cell. Integration may
17 be promoted by inclusion of sequences which promote
18 recombination with the genome in accordance with
19 standard techniques. The nucleic acid may be on an
20 extra-chromosomal vector within the cell, or
21 otherwise identifiably heterologous or foreign to
22 the cell.

23

24 Chemotherapeutic Agents

25

26 As described above, the present invention is based
27 on the surprising demonstration that combining
28 treatment using a death receptor ligand such as the
29 CH11 antibody with a chemotherapeutic agent results
30 in a surprisingly enhanced synergistic therapeutic
31 effect.

32

1 Any suitable chemotherapeutic agent or agents may be
2 used in the present invention. For example, the
3 agent for use in the invention may include but is
4 not limited to: 5-Fluorouracil (5 FU), tomudex (TDX)
5 antifolates, for example RTX or MTA, Doxorubicin,
6 taxol, Leucovorin, Irinotecan, Mitomycin C,
7 Oxaliplatin, Raltitrexed, Tamoxifen or Cisplatin.

8
9 In particularly preferred embodiments, the agent is
10 5-FU or an antifolate. More preferably, the agent
11 is an antifolate. In one preferred embodiment, the
12 agent is MTA.

13
14 **Treatment**

15 "Treatment" includes any regime that can benefit a
16 human or non-human animal. The treatment may be in
17 respect of an existing condition or may be
18 prophylactic (preventative treatment). Treatment may
19 include curative, alleviation or prophylactic
20 effects.

21
22 "Treatment of cancer" includes treatment of
23 conditions caused by cancerous growth and includes
24 the treatment of neoplastic growths or tumours.
25 Examples of tumours that can be treated using the
26 invention are, for instance, sarcomas, including
27 osteogenic and soft tissue sarcomas, carcinomas,
28 e.g., breast-, lung-, bladder-, thyroid-, prostate-,
29 colon-, rectum-, pancreas-, stomach-, liver-,
30 uterine-, cervical and ovarian carcinoma, lymphomas,
31 including Hodgkin and non-Hodgkin lymphomas,
32 neuroblastoma, melanoma, myeloma, Wilms tumor, and

1 leukemias, including acute lymphoblastic leukaemia
2 and acute myeloblastic leukaemia, gliomas and
3 retinoblastomas.
4

5

6 The compositions and methods of the invention may be
7 particularly useful in the treatment of existing
8 cancer and in the prevention of the recurrence of
9 cancer after initial treatment or surgery.

10

11 Administration

12

13 Binding members and chemotherapeutic agents may be
14 administered simultaneously, separately or
15 sequentially.

16

17 Where administered separately or sequentially, they
18 may be administered within any suitable time period
19 e.g. within 1, 2, 3, 6, 12, 24, 48 or 72 hours of
20 each other. In preferred embodiments, they are
21 administered within 6, preferably within 2, more
22 preferably within 1, most preferably within 20
23 minutes of each other. ***Please advise on preferred
24 ranges***

25

26 In a preferred embodiment, they are administered as
27 a pharmaceutical composition, which will generally
28 comprise a suitable pharmaceutical excipient,
29 diluent or carrier selected dependent on the
30 intended route of administration.

31

1 Binding members and chemotherapeutic agents of and
2 for use in the present invention may be administered
3 to a patient in need of treatment via any suitable
4 route. The precise dose will depend upon a number of
5 factors, including the precise nature of the member
6 (e.g. whole antibody, fragment or diabody) and
7 chemotherapeutic agent.

8
9 Some suitable routes of administration include (but
10 are not limited to) oral, rectal, nasal, topical
11 (including buccal and sublingual), vaginal or
12 parenteral (including subcutaneous, intramuscular,
13 intravenous, intradermal, intrathecal and epidural)
14 administration. Intravenous administration is
15 preferred.

16
17 It is envisaged that injections (intravenous) will
18 be the primary route for therapeutic administration
19 of compositions although delivery through a catheter
20 or other surgical tubing is also envisaged. Liquid
21 formulations may be utilised after reconstitution
22 from powder formulations.

23
24 For intravenous, injection, or injection at the site
25 of affliction, the active ingredient will be in the
26 form of a parenterally acceptable aqueous solution
27 which is pyrogen-free and has suitable pH,
28 isotonicity and stability. Those of relevant skill
29 in the art are well able to prepare suitable
30 solutions using, for example, isotonic vehicles such
31 as Sodium Chloride Injection, Ringer's Injection,
32 Lactated Ringer's Injection. Preservatives,

1 stabilisers, buffers, antioxidants and/or other
2 additives may be included, as required.
3

4 Pharmaceutical compositions for oral administration
5 may be in tablet, capsule, powder or liquid form. A
6 tablet may comprise a solid carrier such as gelatin
7 or an adjuvant. Liquid pharmaceutical compositions
8 generally comprise a liquid carrier such as water,
9 petroleum, animal or vegetable oils, mineral oil or
10 synthetic oil. Physiological saline solution,
11 dextrose or other saccharide solution or glycols
12 such as ethylene glycol, propylene glycol or
13 polyethylene glycol may be included.
14

15 The binding member, agent, product or composition
16 may also be administered via microspheres,
17 liposomes, other microparticulate delivery systems
18 or sustained release formulations placed in certain
19 tissues including blood. Suitable examples of
20 sustained release carriers include semipermeable
21 polymer matrices in the form of shaped articles,
22 e.g. suppositories or microcapsules. Implantable or
23 microcapsular sustained release matrices include
24 polylactides (US Patent No. 3, 773, 919; EP-A-
25 0058481) copolymers of L-glutamic acid and gamma
26 ethyl-L-glutamate (Sidman et al, Biopolymers 22(1):
27 547-556, 1985), poly (2-hydroxyethyl-methacrylate)
28 or ethylene vinyl acetate (Langer et al, J. Biomed.
29 Mater. Res. 15: 167-277, 1981, and Langer, Chem.
30 Tech. 12:98-105, 1982). Liposomes containing the
31 polypeptides are prepared by well-known methods: DE
32 3,218, 121A; Epstein et al, PNAS USA, 82: 3688-3692,

1 1985; Hwang et al, PNAS USA, 77: 4030-4034, 1980;
2 EP-A-0052522; E-A-0036676; EP-A-0088046; EP-A-
3 0143949; EP-A-0142541; JP-A-83-11808; US Patent Nos
4 4,485,045 and 4,544,545. Ordinarily, the liposomes
5 are of the small (about 200-800 Angstroms)
6 unilamellar type in which the lipid content is
7 greater than about 30 mol. % cholesterol, the
8 selected proportion being adjusted for the optimal
9 rate of the polypeptide leakage.

10

11 Examples of the techniques and protocols mentioned
12 above and other techniques and protocols which may
13 be used in accordance with the invention can be
14 found in Remington's Pharmaceutical Sciences, 16th
15 edition, Oslo, A. (ed), 1980.

16

17 The binding member, agent, product or composition
18 may be administered in a localised manner to a
19 tumour site or other desired site or may be
20 delivered in a manner in which it targets tumour or
21 other cells. Targeting therapies may be used to
22 deliver the active agents more specifically to
23 certain types of cell, by the use of targeting
24 systems such as antibody or cell specific ligands.
25 Targeting may be desirable for a variety of reasons,
26 for example if the agent is unacceptably toxic, or
27 if it would otherwise require too high a dosage, or
28 if it would not otherwise be able to enter the
29 target cells.

30

31 **Pharmaceutical Compositions**

32

1 As described above, the present invention extends to
2 a pharmaceutical composition for the treatment of
3 cancer, the composition comprising a) a specific
4 binding member which binds to a cell death receptor
5 or a nucleic acid encoding said binding member and
6 (b) a chemotherapeutic agent and (c) a
7 pharmaceutically acceptable excipient, diluent or
8 carrier. Pharmaceutical compositions according to
9 the present invention, and for use in accordance
10 with the present invention may comprise, in addition
11 to active ingredients, a pharmaceutically acceptable
12 excipient, carrier, buffer stabiliser or other
13 materials well known to those skilled in the art.
14 Such materials should be non-toxic and should not
15 interfere with the efficacy of the active
16 ingredient. The precise nature of the carrier or
17 other material will depend on the route of
18 administration, which may be oral, or by injection,
19 e.g. intravenous.

20
21 The formulation may be a liquid, for example, a
22 physiologic salt solution containing non-phosphate
23 buffer at pH 6.8-7.6, or a lyophilised powder.

24 25 Dose

26
27 The binding members, agents, products or
28 compositions are preferably administered to an
29 individual in a "therapeutically effective amount",
30 this being sufficient to show benefit to the
31 individual. The actual amount administered, and
32 rate and time-course of administration, will depend

1 on the nature and severity of what is being treated.
2 As described herein, the concentrations are
3 preferably sufficient to show a synergistic effect.
4 Prescription of treatment, e.g. decisions on dosage
5 etc, is ultimately within the responsibility and at
6 the discretion of general practitioners and other
7 medical doctors, and typically takes account of the
8 disorder to be treated, the condition of the
9 individual patient, the site of delivery, the method
10 of administration and other factors known to
11 practitioners.

12
13 The optimal dose can be determined by physicians
14 based on a number of parameters including, for
15 example, age, sex, weight, severity of the condition
16 being treated, the active ingredient being
17 administered and the route of administration. For
18 example, with respect to binding members, in
19 general, a serum concentration of polypeptides and
20 antibodies that permits saturation of receptors is
21 desirable. A concentration in excess of
22 approximately 0.1nM is normally sufficient. For
23 example, a dose of 100mg/m² of antibody provides a
24 serum concentration of approximately 20nM for
25 approximately eight days.

26
27 As a rough guideline, doses of antibodies may be
28 given in amounts of 1ng/kg- 500mg/kg of patient
29 weight. Equivalent doses of antibody fragments
30 should be used at the same or more frequent
31 intervals in order to maintain a serum level in

1 excess of the concentration that permits saturation
2 of death receptor.

3

4 Doses of the binding members may be given at any
5 suitable dose interval e.g. daily, once, twice or
6 thrice weekly.

7

8 For example, the periods of administration of a
9 humanised antibody could be from 1 bolus injection
10 to weekly administration for up to one year in
11 combination with chemotherapeutic agents. The
12 likely dose is upwards of 1mg/per kg/per patient.

13

14 Doses of chemotherapeutic agent will depend on the
15 factors described above but preferably are
16 administered in doses which are within the normal
17 range or, preferably, at a lower concentration than
18 the normal range, wherein the normal range is the
19 range of concentrations at which the
20 chemotherapeutic agent is usually administered in
21 the absence of other therapeutic agents.

22

23 It is anticipated that in embodiments of the
24 invention the binding members and chemotherapeutic
25 agent could be given in combination with other forms
26 of chemotherapy or indeed radiotherapy.

27

28 Indeed it is believed that the advantages of the
29 invention may also be obtained when using specific
30 binding members of the invention and radiotherapy,
31 even in the absence of chemotherapeutic agents.

32

1 Thus, in a tenth aspect of the invention, there is
2 provided a method of killing cancer cells comprising
3 administration of a therapeutically effective amount
4 of a) a specific binding member which binds to a
5 cell death receptor or a nucleic acid encoding said
6 binding member and (b) radiotherapy treatment.

7
8 In a eleventh aspect, the present invention provides
9 a method of treating cancer comprising
10 administration of a therapeutically effective amount
11 of a) a specific binding member which binds to a
12 cell death receptor or a nucleic acid encoding said
13 binding member and (b) radiotherapy treatment to a
14 mammal in need thereof.

15
16 The specific binding member and the radiotherapy may
17 be administered simultaneously, sequentially or
18 simultaneously. In preferred embodiments of the
19 invention, the chemotherapeutic agent is
20 administered prior to the radiotherapy.

21
22 The invention will now be described further in the
23 following non-limiting examples. Reference is made
24 to the accompanying drawings in which:

25
26 Figure 1A illustrates Northern blot analysis of Fas
27 mRNA expression in MCF-7 cells 48 hours after
28 treatment with no drug (C) or 5 μ M 5-FU. Equal
29 loading was assessed by analysing β -tubulin mRNA
30 expression.

31

1 Figure 1B illustrates Western blot analysis of Fas
2 expression in MCF-7 cells 72 hours after treatment
3 with no drug (C), 5 μ M 5-FU or 25nM RTX. Equal
4 loading was assessed by analysing β -tubulin
5 expression.

6
7 Figure 1C illustrates MTT cell viability assays in
8 MCF-7 cells treated with no drug (control), CH-11
9 alone (250ng/ml), 5-FU alone (5 μ M), or co-treated
10 with 5-FU and CH-11. The decrease in cell viability
11 for the combined treatment was highly synergistic
12 (RI=2.40, $p<0.0005$).

13
14 Figure 1D illustrates MTT cell viability assays in
15 MCF-7 cells treated with no drug (control), CH-11
16 alone (250ng/ml), RTX alone (25nM), or co-treated
17 with RTX and CH-11. The decrease in cell viability
18 for the combined treatment was highly synergistic
19 (RI=2.22, $p<0.0005$).

20
21 Figure 1E illustrates analysis of apoptosis in 5-FU
22 and CH-11 co-treated MCF-7 cells.

23
24 Figure 1F illustrates analysis of apoptosis in RTX
25 and CH-11 co-treated MCF-7 cells. Apoptosis was
26 assessed by analysing the sub-G₁/G₀ fraction of
27 propidium iodide stained cells by flow cytometry.
28 For both the MTT and flow cytometric analyses the
29 cells were pre-treated with each chemotherapeutic
30 drug for 72 hours followed by CH-11 for a further 24
31 hours.

32

1 Figure 2A illustrates Western blot analysis of Fas
2 expression in HCT116p53^{+/+} cells treated with a range
3 of concentrations of 5-FU for 48 hours.

4
5 Figure 2B illustrates MTT cell viability assays in
6 HCT116p53^{+/+} cells treated with no drug (control),
7 CH-11 alone (250ng/ml), 5-FU alone (5 μ M), or co-
8 treated with 5-FU and CH-11. The decrease in cell
9 viability for the combined treatment was synergistic
10 (RI=1.92, $p<0.005$).

11
12 Figure 2C illustrates Western blot analysis of Fas
13 expression in HCT116p53^{+/+} cells treated with a range
14 of concentrations of RTX for 48 hours.

15
16 Figure 2D illustrates MTT cell viability assays in
17 HCT116p53^{+/+} cells treated with no drug (control),
18 CH-11 alone (250ng/ml), RTX alone (50nM), or co-
19 treated with RTX and CH-11. The decrease in cell
20 viability for the combined treatment was highly
21 synergistic (RI=3.44, $p<0.0005$).

22
23 Figure 2E illustrates Western blot analysis of Fas
24 expression in RKO cells treated with a range of
25 concentrations of 5-FU for 48 hours.

26
27 Figure 2F illustrates MTT cell viability assays in
28 RKO cells treated with no drug (control), CH-11
29 alone (250ng/ml), 5-FU alone (5 μ M), or co-treated
30 with 5-FU and CH-11. The decrease in cell viability
31 for the combined treatment was synergistic (RI=1.74,
32 $p<0.005$).

Figure 2G illustrates Western blot analysis of Fas expression in RKO cells treated with a range of concentrations of RTX for 48 hours.

Figure 2H illustrates MTT cell viability assays in RKO cells treated with no drug (control), CH-11 alone (250ng/ml), RTX alone (5nM), or co-treated with RTX and CH-11. The decrease in cell viability for the combined treatment was highly synergistic (RI=2.31, $p<0.0005$). Equal loading of Western blots was assessed by analysing β -tubulin expression. For each combined treatment the cells were pre-treated with chemotherapeutic drug for 72 hours followed by CH-11 for a further 24 hours.

Figure 3A illustrates Western blot analysis of Fas, FasL, procaspase 8 and BID expression in MCF-7 cells treated with IC₅₀ doses of 5-FU (5 μ M) and RTX (25nM) for 72 hours. Equal loading was assessed using a β -tubulin antibody.

Figure 3B illustrates Western blot analysis of Fas, procaspase 8 and BID expression in MCF-7 cells treated no drug (control), CH-11 alone (250ng/ml), 5-FU alone (5 μ M) for 96 hours, or co-treated with 5-FU for 72 hours followed by CH-11 for a further 24 hours. Co-treatment with 5-FU and CH-11 resulted in activation of caspase 8 and BID as indicated by processing of procaspase 8 and full-length BID (lane 4).

1 Figure 3C illustrates Western blot analysis of
2 procaspase 8 and PARP expression in HCT116p53^{+/+}
3 cells treated with no drug (control), 5 μ M 5-FU or
4 50nM RTX alone or in combination with 250ng/ml CH-
5 11.

6
7 Figure 3D illustrates Western blot analysis
8 examining the kinetics of caspase 8 activation and
9 PARP cleavage in MCF-7 cells treated for 72 hours
10 with 5 μ M 5-FU followed by 250ng/ml CH-11 for the
11 indicated times.

12
13 Figure 3E illustrates Western blot analysing Fas,
14 procaspase 8 and PARP expression in MCF-7 cells
15 treated with 5 μ M 5-FU for 72 hours followed by
16 250ng/ml CH-11, 10 μ M IETD-fmk, or a combination of
17 CH-11 and IETD-fmk for 24 hours.

18
19 Figure 4A illustrates tetracycline (tet)-regulated
20 expression of a TS trans-gene in M7TS90 cells.

21
22 Figure 4B illustrates Western blot analysing the
23 effect of TS induction (-tet lanes) on Fas up-
24 regulation in M7TS90 cells in response to treatment
25 with 10 μ M 5-FU, 100nM RTX or 1 μ M MTA for 72 hours.

26
27
28
29 Figure 4C illustrates an MTT assay showing the
30 impact of TS induction (-tet) on viability of M7TS90
31 cells following treatment with 5-FU (10 μ M) or RTX

1 (100nM) in the presence of co-treatment with
2 250ng/ml CH-11.

3

4 Figure 4D illustrates the impact of TS induction on
5 caspase 8 activation and processing of full-length
6 (118kDa) PARP in M7TS90 cells treated with 5-FU
7 (10 μ M), RTX (100nM) or MTA (1 μ M) followed by
8 250ng/ml CH-11.

9

10 Figure 4E illustrates Effect of exogenous TS
11 expression on the induction of apoptosis in M7TS90
12 cells treated with 5-FU (10 μ M) RTX (100nM) or MTA
13 (1 μ M) in the presence of co-treatment with 250ng/ml
14 CH-11. Apoptosis was assessed by analysing the sub-
15 G₁/G₀ fraction of propidium iodide stained cells by
16 flow cytometry. Equal loading of Western blots was
17 assessed by analysing β -tubulin expression. For each
18 combined treatment the cells were pre-treated with
19 chemotherapeutic drug for 72 hours followed by CH-11
20 for a further 24 hours.

21

22 Figure 5A illustrates Western blot analysis of Fas
23 expression in p53 wild type (wt) M7TS90 and p53 null
24 (nl) M7TS90-E6 cells 72 hours after treatment with
25 no drug (Con), 10 μ M 5-FU, 100nM RTX or 1 μ M MTA.

26

27 Figure 5B illustrates MTT cell viability assays in
28 p53 null M7TS90-E6 cells treated with 10 μ M 5-FU,
29 100nM RTX or 1 μ M MTA in combination with 250ng/ml
30 CH-11.

31

1 Figure 5C illustrates Western blot analysis of
2 procaspase 8 and full-length (118kDa) PARP
3 expression in M7TS90 (wt) and M7TS90-E6 (nl) cells
4 treated with 5-FU (10 μ M), RTX (100nM) or MTA (1 μ M)
5 followed by 250ng/ml CH-11.

6
7 Figure 5D illustrates Effect of CH-11 (250ng/ml) on
8 the induction of apoptosis in M7TS90-E6 cells
9 treated with 5-FU (10 μ M) RTX (100nM) or MTA (1 μ M).
10 Apoptosis was assessed by analysing the sub-G₁/G₀
11 fraction of propidium iodide stained cells by flow
12 cytometry. Equal loading of Western blots was
13 assessed by analysing β -tubulin expression. For each
14 combined treatment the cells were pre-treated with
15 chemotherapeutic drug for 72 hours followed by CH-11
16 for a further 24 hours.

17
18 Figure 6A illustrates Western blot analysis of Fas
19 expression in HCT116p53^{-/-} cells treated with a range
20 of concentrations of 5-FU for 48 hours.

21
22 Figure 6B illustrates MTT cell viability assays in
23 HCT116p53^{-/-} cells treated with no drug (control),
24 CH-11 alone (250ng/ml), 5-FU alone (10 μ M), or co-
25 treated with 5-FU and CH-11. The decrease in cell
26 viability for the combined treatment was not
27 synergistic (RI=1.01).

28
29 Figure 6C illustrates Western blot analysis of Fas
30 expression in HCT116p53^{-/-} cells treated with a range
31 of concentrations of RTX for 48 hours.

32

1 Figure 6D illustrates MTT cell viability assays in
2 HCT116p53^{-/-} cells treated with no drug (control),
3 CH-11 alone (250ng/ml), RTX alone (50nM), or co-
4 treated with RTX and CH-11. The decrease in cell
5 viability for the combined treatment was synergistic
6 (RI=1.62, p=0.01).

7
8 Figure 6E illustrates Western blot analysis of Fas
9 expression in H630 cells treated with a range of
10 concentrations of 5-FU for 48 hours.

11
12 Figure 6F illustrates MTT cell viability assays in
13 H630 cells treated with no drug (control), CH-11
14 alone (250ng/ml), 5-FU alone (10μM), or co-treated
15 with 5-FU and CH-11. The decrease in cell viability
16 for the combined treatment was not synergistic
17 (RI=0.99).

18
19 Figure 6G illustrates Western blot analysis of H630
20 cells treated with a range of concentrations of RTX
21 for 48 hours.

22
23 Figure 6H illustrates MTT cell viability assays in
24 H630 cells treated with no drug (control), CH-11
25 alone (250ng/ml), RTX alone (50nM), or co-treated
26 with 5-FU and CH-11. The decrease in cell viability
27 for the combined treatment was synergistic (RI=1.41,
28 p<0.005). Equal loading of Western blots was
29 assessed by analysing β-tubulin expression. For each
30 combined treatment the cells were pre-treated with
31 chemotherapeutic drug for 72 hours followed by CH-11
32 for a further 24 hours.

1

2 MATERIALS AND METHODS

3 Cell Culture. All cells were maintained in 5% CO₂ at
4 37°C. MCF-7, H630 and RKO cells were maintained in
5 DMEM with 10% dialyzed bovine calf serum
6 supplemented with 1mM sodium pyruvate, 2mM L-
7 glutamine and 50µg/ml penicillin/streptomycin (from
8 Life Technologies Inc., Paisley, Scotland). M7TS90
9 cells (6) were maintained in 'MCF-7 medium'
10 supplemented with 1µg/ml puromycin, 1µg/ml
11 tetracycline (from Sigma, Poole, Dorset, England),
12 and 100µg/ml G418 (from Life Technologies Inc).
13 M7TS90-E6 cells (6) were maintained in 'M7TS90
14 medium' supplemented with 200µg/ml hygromycin (Life
15 Technologies Inc). To induce expression of exogenous
16 TS, cells were washed three times in 1xPBS and
17 incubated in growth medium lacking tetracycline.
18 HCT116 p53^{+/+} and p53^{-/-} isogenic human colon cancer
19 cells were kindly provided by Professor Bert
20 Vogelstein (John Hopkins University, Baltimore, MD).
21 HCT116 cell lines were grown in McCoy's 5A medium
22 (GIBCO) supplemented with 10% dialysed foetal calf
23 serum, 50µg/ml penicillin-streptomycin, 2mM L-
24 glutamine and 1mM sodium pyruvate.

25

26 Northern blot analysis. Northern blots were
27 performed as described previously using a cDNA probe
28 complementary to the Fas coding region (7). Equal
29 loading was assessed using a β-tubulin cDNA probe.

30

1 Western Blotting. Western blots were performed as
2 previously described (6). The Fas/CD95, Bcl-2 and
3 BID (Santa Cruz Biotechnology, Santa Cruz, CA),
4 caspase 8 (Oncogene Research Products, Darmstadt,
5 Germany) and PARP (Pharmingen, BD Biosciences,
6 Oxford, England) mouse monoclonal antibodies were
7 used in conjunction with a horseradish peroxidase
8 (HRP)-conjugated sheep anti-mouse secondary antibody
9 (Amersham, Little Chalfont, Buckinghamshire,
10 England). FasL rabbit polyclonal antibody (Santa
11 Cruz Biotechnology) was used in conjunction with an
12 HRP-conjugated donkey anti-rabbit secondary antibody
13 (Amersham). TS sheep monoclonal primary antibody
14 (Rockland, Gilbertsville, PA) was used in
15 conjunction with an HRP-conjugated donkey anti-sheep
16 secondary antibody (Serotech, Oxford, England).
17 Equal loading was assessed using a β -tubulin mouse
18 monoclonal primary antibody (Sigma).

19
20 Cell Viability Assays. Cell viability was assessed
21 by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-
22 diphenyltetrazolium bromide, Sigma) assay (12).
23 Cells were seeded at 2,500 cells per well on 96-well
24 plates 24 hours prior to drug treatment and then
25 treated with a range of concentrations of 5-FU, RTX
26 and MTA for 72 hours, following which time the
27 agonistic Fas monoclonal antibody, CH-11 (MBL,
28 Watertown, MA), was added (10-250ng/ml) for a
29 further 24 hours. MTT (0.5mg/ml) was then added to
30 each well and the cells incubated at 37°C for a
31 further 3 hours. The culture medium was removed and
32 formazan crystals reabsorbed in 200 μ L DMSO. Cell

1 viability was determined by reading the absorbance
2 of each well at 570nm using a 96-well microplate
3 reader (Molecular Devices, Wokingham, England).
4

5 **Flow Cytometric Analysis.** Cells were seeded at 1×10^5
6 per well of a 6-well tissue culture plate. After 24
7 hours, 5-FU, RTX or MTA were added to the medium and
8 the cells cultured for a further 72 hours, after
9 which time 250ng/ml CH-11 was added for 24 hours.
10 DNA content of harvested cells was evaluated after
11 propidium iodide staining of cells using the EPICS
12 XL Flow Cytometer (Coulter, Miami, FL).
13

14 **Statistical Analyses.** The nature of the interaction
15 between the chemotherapeutic drugs and CH-11 was
16 determined by calculating the R index (RI), which
17 was initially described by Kern and later modified
18 by Romanelli (13, 14). The RI is calculated as the
19 ratio of expected cell survival (S_{exp} , defined as the
20 product of the survival observed with drug A alone
21 and the survival observed with drug B alone) to the
22 observed cell survival (S_{obs}) for the combination of
23 A and B ($RI = S_{exp}/S_{obs}$). Synergism is then defined as
24 an RI of greater than unity. Romanelli et al.
25 suggest that a synergistic interaction may be of
26 pharmacological interest when RI values are around
27 2.0 (14). This method was selected because treatment
28 with CH-11 alone had little effect on cell
29 viability, which meant that other methods such as
30 the median effect principle (15) and isobologram
31 methods were not suitable (16). To further assess
32 the statistical significance of the interactions,

1 the inventors designed a univariate ANOVA analysis
2 using the SPSS software package. This was an
3 additive model based on the null hypothesis that
4 there was no interaction between the drugs.

5

6 RESULTS

7 Fas is highly up-regulated in response to 5-FU and
8 RTX. Using DNA microarray profiling, the inventors
9 previously identified the Fas death receptor as
10 being highly up-regulated in response to 5-FU in
11 MCF-7 cells (7). Northern blot analyses confirmed
12 that Fas mRNA was up-regulated in MCF-7 cells 48
13 hours following treatment with an IC_{50} dose (5 μ M) of
14 5-FU (Fig. 1A). Analysis of Fas protein expression
15 in MCF-7 cells revealed that it was up-regulated by
16 ~12-fold 72 hours after treatment with 5-FU (Fig.
17 1B). Fas was also highly up-regulated (by ~7-fold)
18 in response to treatment with an IC_{50} dose (25nM) of
19 RTX (Fig. 1B).

20

21 The agonistic Fas monoclonal antibody CH-11
22 synergistically activates apoptosis in response to
23 5-FU and RTX. To examine the role of the Fas
24 signalling pathway in mediating the response of MCF-
25 7 cells to 5-FU and RTX, the inventors used the
26 agonistic Fas monoclonal antibody CH-11. Cells were
27 treated with IC_{50} doses of each drug for 72 hours,
28 after which time they were treated with 250ng/ml CH-
29 11 for a further 24 hours. Treatment with 5 μ M 5-FU
30 alone resulted in a ~60% reduction in cell viability
31 compared to control (Fig. 1C). Treatment with CH-11
32 alone without prior incubation with 5-FU caused a

1 modest ~6% decrease in cell viability. However,
2 treatment with 5-FU followed by CH-11 was found to
3 result in an ~84% decrease in cell viability. The
4 combined treatment had an RI value of 2.40
5 indicating that the interaction was highly
6 synergistic. This was further confirmed by ANOVA
7 analysis, which indicated that the synergistic
8 interaction between the drugs was highly
9 statistically significant ($p < 0.0005$). Similarly,
10 treatment with 25nM RTX for 72 hours followed by CH-
11 11 for 24 hours produced a highly synergistic
12 decrease in cell viability (RI=2.22, $p < 0.0005$, Fig.
13 1D). An IgM isotype control antibody had no effect
14 on the cell viability of drug-treated cells (data
15 not shown).

16
17 To assess the degree of apoptosis in MCF-7 cells
18 treated with 5-FU and RTX individually, or in
19 combination with CH-11, the inventors carried out
20 flow cytometry of propidium iodide stained cells and
21 analysed the sub-G₁/G₀ apoptotic fraction. Cells were
22 treated with either 5-FU or RTX for 72 hours
23 followed by 250ng/ml CH-11 treatment for 24 hours.
24 The inventors found that CH-11 alone had little
25 effect on apoptosis (Figs. 1E and F). Treatment with
26 5-FU alone for 96 hours resulted in a modest ~2-fold
27 induction of apoptosis in response to 5 μ M 5-FU (Fig.
28 1E). However, addition of CH-11 to 5-FU-treated
29 cells resulted in a dramatic increase in apoptosis,
30 with a ~12-fold induction of apoptosis following co-
31 treatment with 5 μ M 5-FU and CH-11. Similarly, the
32 combination of RTX with CH-11 resulted in dramatic

1 activation of apoptosis, with ~60% of cells in the
2 sub-G₁/G₀ apoptotic phase following combined
3 treatment with 25nM RTX and CH-11 compared to ~11%
4 in untreated control cells, ~16% in cells treated
5 with RTX alone and ~18% in cells treated with CH-11
6 alone (Fig. 1F). The activation of apoptosis by CH-
7 11 in 5-FU and RTX treated cultures was observed
8 across a range of concentrations of each drug (Figs.
9 1E and F), indicating that the synergistic
10 interaction between CH-11 and both drugs was due to
11 activation of apoptosis.

12
13 The inventors next examined the ability of CH-11 to
14 activate apoptosis in other cell lines. Treatment of
15 HCT116p53^{+/+} colon cancer cells with 5-FU resulted in
16 potent up-regulation (>10-fold) of Fas expression
17 after 48 hours (Fig. 2A). Furthermore, treatment
18 with 5 μ M 5-FU followed by 250ng/ml CH-11
19 synergistically decreased cell viability in this
20 line with an RI value of 1.92 (p<0.005). Similarly,
21 RTX treatment dramatically increased Fas expression
22 after 72 hours (Fig. 2C), while treatment with RTX
23 followed by CH-11 resulted in a highly synergistic
24 decrease in cell viability (Fig. 2D, RI=3.44,
25 p<0.0005). The inventors also examined another p53
26 wild type colon cancer cell line, RKO. As was the
27 case with both MCF-7 and HCT116p53^{+/+} cells, both 5-
28 FU and RTX treatment resulted in dramatic Fas up-
29 regulation 48 hours post-treatment (Figs. 3E and F).
30 Furthermore, treatment of RKO cells with 5-FU or RTX
31 followed by CH-11 synergistically decreased cell
32 viability with RI values of 1.74 (p<0.0005) and 2.31

1 (p<0.0005) respectively (Figs. 3F and G). These
2 results indicate that CH-11 not only activates
3 apoptosis of 5-FU- and RTX-treated MCF-7 breast
4 cancer cells, but also of HCT116p53^{+/+} and RKO colon
5 cancer cells. The inventors also found that
6 treatment with the antifolate MTA up-regulated Fas
7 expression and synergistically interacted with CH-11
8 to decrease cell viability in all three cell lines
9 (data not shown).

10

11 **Effect of 5-FU and RTX on Fas signal transduction.**
12 The inventors next examined drug-induced activation
13 of the Fas signalling pathway in response to 5-FU
14 and RTX. Although Fas was highly up-regulated (>10-
15 fold) in MCF-7 cells in response to IC₅₀ doses of
16 either drug, FasL expression was unaffected (Fig.
17 3A). Surprisingly, neither caspase 8, nor its
18 substrate BID were activated in 5-FU- or RTX-treated
19 cells as indicated by a lack of down-regulation of
20 the levels of procaspase 8 or full-length BID (Fig.
21 3A). The inventors subsequently analysed activation
22 of the Fas pathway in MCF-7 cells following co-
23 treatment with 5-FU and CH-11. Fas, procaspase 8 and
24 BID expression levels were determined in cells
25 treated with 5µM 5-FU for 72 hours followed by
26 250ng/ml CH-11 for 24 hours and compared to cells
27 treated with 5-FU alone or CH-11 alone for the
28 appropriate time periods (Fig. 3B). Treatment with
29 CH-11 alone had no effect on Fas, procaspase 8 or
30 BID expression (Fig. 3B, lane 2). As already noted,
31 treatment with 5-FU alone resulted in dramatic up-
32 regulation of Fas, but had no effect on procaspase 8

1 or BID expression, indicating that neither molecule
2 was activated (Fig. 3B, lane 3). However, treatment
3 of MCF-7 cells with 5-FU and CH-11 resulted in a
4 dramatic activation of both caspase 8 and BID as
5 indicated by complete loss of procaspase 8 and full-
6 length BID expression in these cells (Fig. 3B, lane
7 4). Similarly, in HCT116p53^{+/+} cells activation of
8 caspase 8 was only observed following co-treatment
9 with either 5-FU and CH-11 or RTX and CH-11 (Fig.
10 3C). Furthermore, cleavage of PARP (poly(ADP) ribose
11 polymerase), a hallmark of apoptosis, was only
12 observed in HCT116p53^{+/+} cells co-treated with each
13 drug and CH-11.

14
15 The inventors next compared the kinetics of caspase
16 8 activation with cleavage of PARP. Six hours after
17 addition of CH-11 to MCF-7 cells pre-treated for 72
18 hours with 5 μ M 5-FU, procaspase 8 levels were
19 reduced by ~3-fold compared to time zero (Fig. 3D).
20 This coincided with PARP cleavage, which is
21 indicative of cells undergoing apoptosis. Thus,
22 activation of caspase 8 coincided with the onset of
23 apoptosis. Twelve and 18 hours following CH-11
24 treatment, the levels of procaspase 8 had fallen to
25 less than 5% of that observed at time zero,
26 indicating potent activation of caspase 8. The
27 inventors further examined the relationship between
28 caspase 8 activation and apoptosis using the
29 specific caspase 8 inhibitor IETD-fmk. Cells were
30 pre-treated with 5 μ M 5-FU for 72 hours followed by
31 250ng/ml CH-11, 10 μ M IETD-fmk, or a combination of
32 CH-11 and IETD-fmk for 24 hours. Fas was highly up-

1 regulated in all treatment groups (Fig. 3D). As
2 noted above, the combination of 5-FU and CH-11
3 resulted in a dramatic activation of caspase 8 and
4 PARP cleavage (Fig. 3E, lane 2). Addition of the
5 caspase 8 inhibitor had no effect on protein
6 expression in cells treated with 5-FU alone (Fig.
7 3E, lane 3). However, IETD-fmk blocked processing of
8 procaspase 8 in cells co-treated with 5-FU and CH-11
9 (Fig. 3E, lane 4). This result indicates that
10 caspase 8 activity is necessary for procaspase 8
11 processing at the DISC and is consistent with the
12 induced proximity model proposed for caspase 8
13 activation (17). Significantly, blocking caspase 8
14 activation also inhibited PARP cleavage in 5-FU/CH-
15 11 co-treated cells, indicating that apoptosis of
16 these cells is dependent on caspase 8 activation.
17
18 Effect of TS induction on the synergy between CH-11
19 and 5-FU, RTX and MTA. Treatment with 5-FU and TS-
20 targeted antifolates has been shown to acutely
21 increase TS expression, most likely through
22 disruption of a negative feedback mechanism in which
23 TS binds to and inhibits translation of its own mRNA
24 (18). This constitutes a potential mechanism of
25 resistance as TS induction would facilitate recovery
26 of enzymatic activity. The inventors therefore
27 examined the effect of inducible TS expression on 5-
28 FU and antifolate-mediated up-regulation of Fas and
29 the synergistic interaction between CH-11 and each
30 drug. To do this, the inventors used the MCF-7-
31 derived M7TS90 cell line (6), in which transcription
32 of a TS trans-gene is activated following withdrawal

1 of tetracycline (tet) from the culture medium (Fig.
2 4A). In agreement with the inventors' previous
3 findings, TS induction in the M7TS90 cell line
4 abrogated RTX- and MTA-, but not 5-FU-mediated up-
5 regulation of Fas (Fig. 4B) (6). Furthermore,
6 induction of the TS trans-gene had little effect on
7 the synergistic interaction between 5-FU and CH-11
8 (Fig. 4C). However, TS induction completely
9 abolished the synergistic decrease in cell viability
10 caused by the combination of both 100nM RTX and CH-
11 11 and 1 μ M MTA and CH-11 (Fig. 4C).

12
13 The inventors next assessed the effect of inducible
14 TS on caspase 8 activation. The inventors found that
15 TS induction abrogated caspase 8 activation in
16 response to co-treatment with both antifolates and
17 CH-11, but had no effect on caspase 8 activation in
18 response to co-treatment with 5-FU and CH-11 (Fig.
19 4D). Similarly, TS induction abrogated processing of
20 PARP in cells co-treated with the antifolates and
21 CH-11, but not in cells co-treated with 5-FU and CH-
22 11 (Fig. 4D). The differential effects of TS
23 induction on apoptosis of 5-FU- and antifolate-
24 treated M7TS90 cells was further analysed by flow
25 cytometry by assessing of the sub-G₀/G₁ fraction in
26 cells co-treated with drug and CH-11. Co-treatment
27 with 5-FU and CH-11 resulted in a dramatic ~20-fold
28 induction of apoptosis in M7TS90 cells that was only
29 modestly reduced to ~17-fold when TS was induced
30 (Fig. 4E). In contrast, RTX and CH-11 co-treatment
31 resulted in a ~15-fold increase in the apoptotic
32 fraction, which was reduced to ~5-fold by TS

1 induction (Fig. 4E). Similarly, combined treatment
2 with MTA and CH-11 resulted in a dramatic ~26-fold
3 induction of apoptosis that was almost completely
4 abolished by inducible TS expression (Fig. 4E).
5 These results indicate that the activation of Fas-
6 mediated apoptosis in antifolate-treated cells was
7 highly dependent on TS expression levels. In
8 contrast, the 5-FU/CH-11 interaction was relatively
9 insensitive to TS induction in this cell line,
10 suggesting that non-TS-directed effects were
11 primarily responsible for 5-FU cytotoxicity in these
12 cells.
13

1 Effect of p53 inactivation on the synergy between
2 CH-11 and 5-FU, RTX and MTA. The inventors next
3 examined the role of p53 in the observed synergy
4 between CH-11 and each drug. p53 has been reported
5 to be an important regulator of Fas expression, both
6 transcriptionally (19) and post-transcriptionally
7 (20). The inventors previously described the
8 generation of p53 null M7TS90-E6 cells by
9 transfection of M7TS90 cells with human papilloma
10 virus (HPV)-E6 (6). Treatment of these p53 null
11 M7TS90-E6 cells with 10 μ M 5-FU, 100nM RTX or 1 μ M MTA
12 did not result in Fas up-regulation (Fig. 5A).
13 Furthermore, in contrast to the parental line, the
14 combination of 5-FU and CH-11 did not
15 synergistically decrease cell viability (RI=0.97,
16 Fig. 5B). Similarly, inactivation of p53 also
17 abolished the synergy between RTX and CH-11 and
18 between MTA and CH-11 (RI=0.85 and 1.02
19 respectively, Fig. 5B).

20
21 The inventors further examined the effects of p53
22 inactivation on drug sensitivity by comparing
23 caspase 8 activation in the p53 wild type and null
24 isogenic M7TS90 lines. Activation of caspase 8 was
25 not observed in the p53 null M7TS90-E6 cells co-
26 treated with each drug and CH-11 (Fig. 5C). In
27 contrast, caspase 8 was potently activated in the
28 parental p53 wild type cell line in response to each
29 co-treatment (Fig. 5C). Inactivation of p53 also
30 completely attenuated PARP cleavage in response to
31 co-treatment with 5-FU and CH-11 (Fig. 5C). However,
32 processing of PARP was evident in p53 null cells

1 treated with both the RTX/CH-11 and MTA/CH-11
2 combinations, although to a lesser extent than in
3 the p53 wild type line (Fig. 5C). As caspase 8 was
4 not activated, this suggests that antifolate-
5 mediated PARP cleavage in the p53 null cells was not
6 due to activation of Fas-mediated apoptosis by CH-
7 11. Indeed, the inventors found that PARP was also
8 processed in the p53 null cell line in response to
9 treatment with either RTX alone or MTA alone (data
10 not shown). These results indicate that treatment
11 with the antifolates activated p53- and Fas-
12 independent apoptosis in M7TS90-E6 cells. This was
13 further confirmed by flow cytometric analysis of
14 apoptosis in the p53 null cell line. RTX (100nM) and
15 MTA (1µM) significantly induced apoptosis of M7TS90-
16 E6 cells by ~8-fold and ~6-fold respectively 96
17 hours after drug treatment (Fig. 5D). In contrast,
18 little apoptosis was observed in M7TS90-E6 cells
19 following treatment with 10µM 5-FU (Fig. 5D).
20 Importantly, CH-11 had no significant effect on
21 apoptosis induced by any of the drugs in the p53
22 null cell line.

23

24 The inventors extended their studies into the role
25 of p53 in regulating antimetabolite-induced Fas-
26 mediated apoptosis by examining the interaction
27 between these drugs and CH-11 in the p53 null
28 HCT116p53^{-/-} cell line. This cell line was derived
29 from the HCT116p53^{+/+} cell line by somatic knock-out
30 of both p53 alleles (21). Compared to the p53 wild
31 type cell line, there was very little Fas induction
32 in response to 5-FU (Fig. 6A) and RTX (Fig. 6C) in

1 the HCT116p53^{-/-} cell line, with an approximate 2-3-
2 fold induction of Fas expression observed in
3 response to 10 μ M 5-FU and 50nM RTX. Furthermore, no
4 synergistic interaction was observed between 5-FU
5 and CH-11 in the p53 null cell line (RI=1.01, Fig.
6 6B). Interestingly, a significant synergistic
7 interaction was still observed between RTX and CH-11
8 in HCT116p53^{-/-} cells (RI=1.62, p=0.01, Fig. 6D),
9 although this was significantly less synergistic
10 than the interaction observed in the p53 wild type
11 parental line (Fig. 2D, RI=3.44, p<0.0005). This
12 suggests that RTX-mediated sensitization of HCT116
13 cells to CH-11 is not wholly p53-dependent.

14
15 The role of p53 in mediating Fas-mediated apoptosis
16 was further examined in the p53 mutant H630 colon
17 cancer cell line. Similar to the p53 null cell
18 lines, Fas expression was not significantly
19 altered in H630 cells in response to 5-FU (Fig. 6E)
20 or RTX (Fig. 6G). No synergistic decrease in cell
21 viability was observed between 5-FU and CH-11 (Fig.
22 6F, RI=0.99), however, a statistically significant
23 synergistic interaction was observed between RTX and
24 CH-11 (Fig. 6H, RI=1.64, p<0.0005). This interaction
25 was observed despite the lack of any apparent up-
26 regulation of Fas in response to this agent,
27 suggesting that Fas expression is not the sole
28 determinant of sensitivity to CH-11 in this cell
29 line.

30 The inventors have observed similar synergistic
31 interactions between anti-Fas monoclonal antibody
32 and both TDX and oxaliplatin (data not shown) in

1 MCF-7 and HCT116 cell line models. Fas-targeted
2 antibodies may thus be used to stimulate apoptosis
3 in chemosensitised cancer cells.
4
5

6 DISCUSSION

7 The inventors have found that the Fas death receptor
8 is highly up-regulated in response to 5-FU and the
9 TS-targeted antifolates RTX and MTA in MCF-7 breast
10 cancer and HCT116p53^{+/+} and RKO colon cancer cells.
11 However, this was in itself not sufficient to
12 activate caspase 8. To mimic the effects of immune
13 effector cells in their *in vitro* model, the
14 inventors used the agonistic Fas monoclonal antibody
15 CH-11. The inventors found that CH-11 potently
16 activated Fas-mediated cell death in 5-FU- and
17 antifolate-treated cells. Furthermore, the
18 interaction between CH-11 and each drug was highly
19 synergistic. The inventors' results suggest that the
20 Fas signalling pathway is an important mediator not
21 only of 5-FU-induced cell death, but also of
22 antifolate-induced cell death.
23

24 The inventors found that although FasL was not
25 induced following drug treatment, it was highly
26 expressed in MCF-7 cells. Many tumour cells
27 overexpress FasL, and it has been postulated that
28 tumour FasL induces apoptosis of Fas-sensitive
29 immune effector cells, thereby inhibiting the
30 antitumor immune response. This hypothesis has been
31 supported by both *in vitro* and *in vivo* studies (24,
32 25). The strategy of overexpressing FasL requires

1 that the tumour cells develop resistance to Fas-
2 mediated apoptosis to prevent autocrine and
3 paracrine induction of tumour cell death. Fas
4 signalling may be inhibited by a Fas splice variant
5 soluble Fas (sFas), which is a secreted protein that
6 lacks the transmembrane domain of full-length Fas
7 and may inhibit binding of FasL to Fas (26).
8 Similarly, the Fas decoy receptor DcR3 is another
9 secreted protein that binds to FasL with high
10 affinity inhibiting its interaction with Fas (27).
11 Downstream of Fas ligation, c-FLIP (FLICE-inhibitory
12 protein) and FAP-1 (Fas-associated phosphatase-1)
13 can inhibit caspase 8 recruitment and activation at
14 the Fas DISC (28, 29). The lack of caspase 8
15 activation in response to treatment with 5-FU and
16 the antifolates suggests that Fas-mediated apoptosis
17 may be inhibited in MCF-7, HCT116 and RKO cancer
18 cells. However, co-treatment with CH-11 was
19 sufficient to overcome this resistance and activate
20 Fas-mediated apoptosis.

21
22 The inventors' findings raise the possibility of
23 using antimetabolite drugs in combination with anti-
24 Fas antibodies as a novel anticancer strategy.
25 Targeting Fas may be particularly useful against
26 tumour cells that overexpress FasL and Fas pathway
27 inhibitors, and which thereby evade Fas-mediated
28 elimination by immune cells. However, systemic
29 treatment with Fas antibodies or rFasL in mouse
30 models has been shown to cause severe damage to
31 liver and other organs (31). Some recent studies
32 have focussed on local administration of rFasL, or

1 the use of FasL-expressing vectors as gene therapy
2 to overcome systemic toxicity (31). In addition, a
3 novel agonistic Fas-targeted antibody HFE7A has been
4 developed recently that was not hepatotoxic in
5 murine models, suggesting that it may be possible to
6 develop less toxic Fas-targeted antibodies (32).

7
8 Treatment with TS inhibitors has been shown to
9 acutely induce TS expression in cell lines and
10 tumours (18, 33). Furthermore, pre-clinical and
11 clinical studies have found that TS is a key
12 determinant of sensitivity to 5-FU, with high TS
13 expression correlating with increased resistance (1,
14 34). The inventors therefore examined the effect of
15 elevated TS expression on activation of Fas-mediated
16 apoptosis in 5-FU- and antifolate-treated cells
17 using a tetracycline-regulated TS expression system
18 (M7TS90). Interestingly, the inventors found that
19 activation of apoptosis by CH-11 in response to 5-FU
20 was not affected by increased TS expression. In
21 contrast, TS induction completely abrogated the
22 synergistic interaction between both RTX and CH-11
23 and MTA and CH-11. These findings correlated with
24 Fas expression, the up-regulation of which was
25 almost completely abrogated by TS induction in RTX-
26 and MTA-treated cells, but not 5-FU-treated cells.
27 These results indicate that the primary locus of 5-
28 FU cytotoxicity in this cell line was not TS
29 inhibition. Indeed, the inventors' previous studies
30 have suggested that misincorporation of
31 fluoronucleotides into RNA was the primary cytotoxic
32 effect of 5-FU in this line (6). Thus, despite

1 expressing high levels of TS, certain tumours may
2 still be sensitised to Fas-mediated apoptosis by 5-
3 FU. However, high TS expression is likely to inhibit
4 Fas-mediated apoptosis in response to folate-based
5 TS inhibitors.

6
7 Several pre-clinical studies have demonstrated that
8 loss of p53 function reduces cellular sensitivity to
9 5-FU (6, 21). Furthermore, a number of clinical
10 studies have found that p53 mutations correlated
11 with resistance to 5-FU, although other studies
12 found no such association (34). The inventors
13 assessed the effect of p53 inactivation on drug-
14 induced Fas-mediated apoptosis in two p53 wild type
15 and null isogenic cell line pairs: the MCF-7-derived
16 M7TS90 and M7TS90-E6 lines, and the HCT116p53^{+/+} and
17 HCT116p53^{-/-} lines. p53 inactivation attenuated Fas
18 up-regulation in response to both drugs in both cell
19 lines and inhibited the activation of apoptosis by
20 CH-11 in 5-FU- and antifolate-treated cells;
21 indicating that p53 is an important determinant of
22 Fas-mediated apoptosis in response to these agents.
23 Interestingly, some synergy was still observed
24 between RTX and CH-11 in the HCT116p53^{-/-} cell line,
25 although it was significantly reduced compared to
26 the p53 wild type cell line. The inventors also
27 examined activation of Fas-mediated apoptosis in
28 response to the antimetabolites in the p53 mutant
29 H630 colon cancer cell line. Similar to the
30 HCT116p53^{-/-} cell line, little Fas induction was
31 observed following drug treatment and no synergy was
32 observed between 5-FU and CH-11. However, a

1 statistically significant synergistic interaction
2 was again observed between RTX and CH-11. The
3 inventors' results surprisingly suggest that RTX
4 (but not 5-FU) can sensitize at least some cancer
5 cell lines with non-functional p53 to Fas-mediated
6 apoptosis. Furthermore, this effect appears to be
7 independent of Fas up-regulation, suggesting that
8 factors other than increased Fas expression
9 contribute to the sensitisation of tumour cells to
10 Fas-mediated apoptosis in response to this agent.
11

12 The inventors' data suggest that tumours with
13 mutated p53 would be more resistant to Fas-mediated
14 apoptosis in response to antimetabolites, in
15 particular 5-FU. However, the discriminatory p53
16 mutants Pro-175 and Ala-143 have been shown to
17 transcriptionally up-regulate Fas expression (35),
18 suggesting that certain p53 mutant tumours may be
19 sensitised to Fas-mediated cell death by
20 chemotherapy.
21

22 In conclusion, the inventors have found that the
23 agonistic Fas monoclonal antibody CH-11 dramatically
24 increases the apoptotic response to 5-FU and TS-
25 targeted antifolates in MCF-7, HCT116p53^{+/+} and RKO
26 cells. Induction of exogenous TS abrogated this
27 synergistic interaction for the antifolates but not
28 5-FU, however, the extent of the interaction was
29 highly p53-dependent for each drug. The inventors'
30 findings suggest that the Fas signalling pathway is
31 an important regulator of 5-FU- and antifolate-
32 mediated cell death and that targeting the Fas

1 pathway in conjunction with either 5-FU or
2 antifolates may have therapeutic potential.

3
4 The inventors have observed similar synergistic
5 interactions between anti-Fas monoclonal antibody
6 and both TDX (Fig.6) and oxaliplatin (data not
7 shown) in MCF-7 and HCT116 cell line models. Fas-
8 targeted antibodies may thus be used to stimulate
9 apoptosis in chemosensitised cancer cells.

10
11 All documents referred to in this specification are
12 herein incorporated by reference. Various
13 modifications and variations to the described
14 embodiments of the inventions will be apparent to
15 those skilled in the art without departing from the
16 scope and spirit of the invention. Although the
17 invention has been described in connection with
18 specific preferred embodiments, it should be
19 understood that the invention as claimed should not
20 be unduly limited to such specific embodiments.
21 Indeed, various modifications of the described modes
22 of carrying out the invention which are obvious to
23 those skilled in the art are intended to be covered
24 by the present invention.

25

26 REFERENCES

- 27 1. Longley, D. B., Harkin, D. P., and Johnston, P.
28 G. 5-fluorouracil: mechanisms of action and
29 clinical strategies. Nat Rev Cancer, 3: 330-
30 338, 2003.
31 2. Hughes, L. R., Stephens, T. C., Boyle, F. T.,
32 and Jackman, A. L. Raltitrexed (TomudexTM), a

- 1 highly polyglutamatable antifolate thymidylate
2 synthase inhibitor, p. 147-165: Humana press,
3 1999.
- 4 3. Shih, C., Chen, V. J., Gossett, L. S., Gates,
5 S. B., MacKellar, W. C., Habeck, L. L.,
6 Shackelford, K. A., Mendelsohn, L. G., Soose,
7 D. J., Patel, V. F., Andis, S. L., Bewley, J.
8 R., Rayl, E. A., Moroson, B. A., Beardsley, G.
9 P., Kohler, W., Ratnam, M., and Schultz, R. M.
10 LY231514, a pyrrolo[2,3-d]pyrimidine-based
11 antifolate that inhibits multiple folate-
12 requiring enzymes. Cancer Res, 57: 1116-1123,
13 1997.
- 14 4. Aherne, G. W., Hardcastle, A., Raynaud, F., and
15 Jackman, A. L. Immunoreactive dUMP and TTP
16 pools as an index of thymidylate synthase
17 inhibition; effect of tomudex (ZD1694) and a
18 nonpolyglutamated quinazoline antifolate
19 (CB30900) in L1210 mouse leukaemia cells.
20 Biochem Pharmacol, 51: 1293-1301, 1996.
- 21 5. Longley, D. B., Ferguson, P. R., Boyer, J.,
22 Latif, T., Lynch, M., Maxwell, P., Harkin, D.
23 P., and Johnston, P. G. Characterization of a
24 thymidylate synthase (TS)-inducible cell line:
25 a model system for studying sensitivity to TS-
26 and non-TS-targeted chemotherapies. Clin Cancer
27 Res, 7: 3533-3539, 2001.
- 28 6. Longley, D. B., Boyer, J., Allen, W. L., Latif,
29 T., Ferguson, P. R., Maxwell, P. J., McDermott,
30 U., Lynch, M., Harkin, D. P., and Johnston, P.
31 G. The role of thymidylate synthase induction
32 in modulating p53-regulated gene expression in

- 1 response to 5-fluorouracil and antifolates.
2 Cancer Res, 62: 2644-2649, 2002.
- 3 7. Maxwell, P. J., Longley, D. B., Latif, T.,
4 Boyer, J., Allen, W., Lynch, M., McDermott, U.,
5 Harkin, D. P., Allegra, C. J., and Johnston, P.
6 G. Identification of 5-fluorouracil-inducible
7 target genes using cDNA microarray profiling.
8 Cancer Research, 63, 2003.
- 9 12. Mosmann, T. Rapid colorimetric assay for
10 cellular growth and survival: application to
11 proliferation and cytotoxicity assays. J
12 Immunol Methods, 65: 55-63, 1983.
- 13 13. Kern, D. H., Morgan, C. R., and Hildebrand-
14 Zanki, S. U. In vitro pharmacodynamics of 1-
15 beta-D-arabinofuranosylcytosine: synergy of
16 antitumor activity with cis-
17 diamminedichloroplatinum(II). Cancer Res, 48:
18 117-121, 1988.
- 19 14. Romanelli, S., Perego, P., Pratesi, G.,
20 Carenini, N., Tortoreto, M., and Zunino, F. In
21 vitro and in vivo interaction between cisplatin
22 and topotecan in ovarian carcinoma systems.
23 Cancer Chemother Pharmacol, 41: 385-390, 1998.
- 24 15. Chou, T. C. and Talalay, P. Quantitative
25 analysis of dose-effect relationships: the
26 combined effects of multiple drugs or enzyme
27 inhibitors. Adv Enzyme Regul, 22: 27-55, 1984.
- 28 16. Kano, Y., Ohnuma, T., Okano, T., and Holland,
29 J. F. Effects of vincristine in combination
30 with methotrexate and other antitumor agents in
31 human acute lymphoblastic leukemia cells in
32 culture. Cancer Res, 48: 351-356, 1988.

- 1 17. Salvesen, G. S. and Dixit, V. M. Caspase
2 activation: the induced-proximity model. Proc
3 Natl Acad Sci U S A, 96: 10964-10967, 1999.
- 4 18. Chu, E., Koeller, D. M., Johnston, P. G., Zinn,
5 S., and Allegra, C. J. Regulation of
6 thymidylate synthase in human colon cancer
7 cells treated with 5-fluorouracil and
8 interferon-gamma. Mol Pharmacol, 43: 527-533,
9 1993.
- 10 19. Muller, M., Wilder, S., Bannasch, D., Israeli,
11 D., Lehlbach, K., Li-Weber, M., Friedman, S.
12 L., Galle, P. R., Stremmel, W., Oren, M., and
13 Krammer, P. H. p53 activates the CD95 (APO-
14 1/Fas) gene in response to DNA damage by
15 anticancer drugs. J Exp Med, 188: 2033-2045,
16 1998.
- 17 20. Bennett, M., Macdonald, K., Chan, S. W., Luzio,
18 J. P., Simari, R., and Weissberg, P. Cell
19 surface trafficking of Fas: a rapid mechanism
20 of p53-mediated apoptosis. Science, 282: 290-
21 293, 1998.
- 22 21. Bunz, F., Hwang, P. M., Torrance, C., Waldman,
23 T., Zhang, Y., Dillehay, L., Williams, J.,
24 Lengauer, C., Kinzler, K. W., and Vogelstein,
25 B. Disruption of p53 in human cancer cells
26 alters the responses to therapeutic agents. J
27 Clin Invest, 104: 263-269, 1999.
- 28 24. O'Connell, J., Bennett, M. W., O'Sullivan, G.
29 C., Collins, J. K., and Shanahan, F. The Fas
30 counterattack: a molecular mechanism of tumor
31 immune privilege. Mol Med, 3: 294-300, 1997.

- 1 25. Bennett, M. W., O'Connell, J., O'Sullivan, G.
2 C., Brady, C., Roche, D., Collins, J. K., and
3 Shanahan, F. The Fas counterattack in vivo:
4 apoptotic depletion of tumor-infiltrating
5 lymphocytes associated with Fas ligand
6 expression by human esophageal carcinoma. *J*
7 *Immunol*, 160: 5669-5675, 1998.
- 8 26. Ruberti, G., Cascino, I., Papoff, G., and
9 Eramo, A. Fas splicing variants and their
10 effect on apoptosis. *Adv Exp Med Biol*, 406:
11 125-134, 1996.
- 12 27. Pitti, R. M., Marsters, S. A., Lawrence, D. A.,
13 Roy, M., Kischkel, F. C., Dowd, P., Huang, A.,
14 Donahue, C. J., Sherwood, S. W., Baldwin, D.
15 T., Godowski, P. J., Wood, W. I., Gurney, A.
16 L., Hillan, K. J., Cohen, R. L., Goddard, A.
17 D., Botstein, D., and Ashkenazi, A. Genomic
18 amplification of a decoy receptor for Fas
19 ligand in lung and colon cancer. *Nature*, 396:
20 699-703, 1998.
- 21 28. Krueger, A., Baumann, S., Krammer, P. H., and
22 Kirchhoff, S. FLICE-inhibitory proteins:
23 regulators of death receptor-mediated
24 apoptosis. *Mol Cell Biol*, 21: 8247-8254, 2001.
- 25 29. Sato, T., Irie, S., Kitada, S., and Reed, J. C.
26 FAP-1: a protein tyrosine phosphatase that
27 associates with Fas. *Science*, 268: 411-415,
28 1995.
- 29 30. Jiang, S., Song, M. J., Shin, E. C., Lee, M.
30 O., Kim, S. J., and Park, J. H. Apoptosis in
31 human hepatoma cell lines by chemotherapeutic

- 1 drugs via Fas-dependent and Fas-independent
2 pathways. *Hepatology*, 29: 101-110, 1999.
- 3 31. Timmer, T., de Vries, E. G., and de Jong, S.
4 Fas receptor-mediated apoptosis: a clinical
5 application? *J Pathol*, 196: 125-134, 2002.
- 6 32. Ichikawa, K., Yoshida-Kato, H., Ohtsuki, M.,
7 Ohsumi, J., Yamaguchi, J., Takahashi, S., Tani,
8 Y., Watanabe, M., Shiraishi, A., Nishioka, K.,
9 Yonehara, S., and Serizawa, N. A novel murine
10 anti-human Fas mAb which mitigates
11 lymphadenopathy without hepatotoxicity. *Int*
12 *Immunol*, 12: 555-562, 2000.
- 13 33. Swain, S. M., Lippman, M. E., Egan, E. F.,
14 Drake, J. C., Steinberg, S. M., and Allegra, C.
15 J. Fluorouracil and high-dose leucovorin in
16 previously treated patients with metastatic
17 breast cancer. *J Clin Oncol*, 7: 890-899, 1989.
- 18 34. Longley, D. B., McDermott, U., and Johnston, P.
19 G. Clinical significance of prognostic and
20 predictive markers in colorectal cancer.
21 *Pharmacogenomics J*, 2: 209-216, 2002.
- 22 35. Munsch, D., Watanabe-Fukunaga, R., Bourdon, J.
23 C., Nagata, S., May, E., Yonish-Rouach, E., and
24 Reisdorf, P. Human and mouse Fas (APO-1/CD95)
25 death receptor genes each contain a p53-
26 responsive element that is activated by p53
27 mutants unable to induce apoptosis. *J Biol*
28 *Chem*, 275: 3867-3872, 2000.
- 29
30
31
32

60

1 **Claims**

- 2
- 3 1. Use of (a) a specific binding member which
- 4 binds to a cell death receptor or a nucleic
- 5 acid encoding said binding member and (b) a
- 6 chemotherapeutic agent in the preparation of a
- 7 medicament for treating cancer.
- 8
- 9 2. The use according to claim 1 wherein the cancer
- 10 is one or more of colorectal, breast , ovarian,
- 11 cervical, gastric, lung, liver, skin and
- 12 myeloid (e.g. bone marrow) cancer.
- 13
- 14 3. The use according to claim 1 or claim 2 wherein
- 15 the binding member is an antibody or a fragment
- 16 thereof.
- 17
- 18 4. The use according to any one of the preceding
- 19 claims wherein the death receptor is FAS.
- 20
- 21 5. The use according to any one of the preceding
- 22 claims wherein the binding member is the anti-
- 23 FAS antibody CH11.
- 24
- 25 6. The use according to any one of the preceding
- 26 claims wherein the binding member comprises at
- 27 least one human constant region.
- 28
- 29 7. The use according to any one of the preceding
- 30 claims wherein, wherein said active agent is 5-
- 31 Fluorouracil or an antifolate.
- 32

- 1 8. The use according to claim 7 wherein said
2 active agent is MTA.
3
- 4 9. A method of killing cancer cells comprising
5 administering a therapeutically effective
6 amount of a) a specific binding member which
7 binds to a cell death receptor or a nucleic
8 acid encoding said binding member and (b) a
9 chemotherapeutic agent.
10
- 11 10. A method of treating cancer comprising
12 administration of a therapeutically effective
13 amount of a) a specific binding member which
14 binds to a cell death receptor or a nucleic
15 acid encoding said binding member and (b) a
16 chemotherapeutic agent to a mammal in need
17 thereof.
18
- 19
- 20 11. The method according to claim 9 or claim 10
21 wherein the cancer is one or more of
22 colorectal, breast , ovarian, cervical,
23 gastric, lung, liver, skin and myeloid (e.g.
24 bone marrow) cancer.
25
- 26 12. The method according to claim 9, 10 or 11
27 wherein the binding member is an antibody or a
28 fragment thereof.
29
- 30 13. The method according to any one of claims 9 to
31 12 wherein the death receptor is FAS.
32

- 1 14. The method according to any one of claims 9 to
2 13 wherein the binding member is the anti-FAS
3 antibody CH11.
4
- 5 15. The method according to any one of claims 9 to
6 13 wherein the binding member comprises at
7 least one human constant region.
8
- 9 16. The method according to any one of claims 9 to
10 15 wherein, wherein said active agent is 5-
11 Fluorouracil or an antifolate.
12
- 13 17. The method according to claim 16 wherein said
14 active agent is MTA.
15
- 16 18. A product comprising a) a specific binding
17 member which binds to a cell death receptor or
18 a nucleic acid encoding said binding member and
19 (b) a chemotherapeutic agent as a combined
20 preparation for the simultaneous, separate or
21 sequential use in the treatment of cancer.
22
- 23 19. A pharmaceutical composition for the treatment
24 of cancer, wherein the composition comprises a)
25 a specific binding member which binds to a cell
26 death receptor or a nucleic acid encoding said
27 binding member and (b) a chemotherapeutic agent
28 and (c) a pharmaceutically acceptable
29 excipient, diluent or carrier.
30
- 31 20. The product according to claim 18 or the
32 pharmaceutical composition according to claim

- 1 19 wherein the cancer is one or more of
2 colorectal, breast , ovarian, cervical,
3 gastric, lung, liver, skin and myeloid (e.g.
4 bone marrow) cancer.
5
6 21. The product according to claim 18 or claim 20
7 or the pharmaceutical composition according to
8 claim 19 or claim 20 wherein the binding member
9 is an antibody or a fragment thereof.
10
11 22. The product according to claim 18 or claim 20
12 or 21 or the pharmaceutical composition
13 according to claim 19 or claim 20 or 21 wherein
14 the death receptor is FAS.
15
16 23. The product according to claim 18 or any one of
17 claims 20 to 22 or the pharmaceutical
18 composition according to claim 19 or or any one
19 of claims 20 to 22 wherein the binding member
20 is the anti-FAS antibody CH11.
21
22 24. The product according to claim 18 or any one of
23 claims 20 to 23 or the pharmaceutical
24 composition according to claim 19 or or any one
25 of claims 20 to 23 wherein the binding member
26 comprises at least one human constant region.
27
28 25. The product according to claim 18 or any one of
29 claims 20 to 24 or the pharmaceutical
30 composition according to claim 19 or or any one
31 of claims 20 to 24 wherein, wherein said active

64

1 agent is 5-Fluorouracil or an antifolate.

2

3 26. The product or pharmaceutical composition
4 according to claim 25 wherein said active agent
5 is MTA.

6

7 27. A kit for the treatment of cancer, said
8 kit comprising:

9 a) a specific binding member which binds to a
10 cell death receptor or a nucleic acid encoding
11 said binding member and (b) a chemotherapeutic
12 agent and
13 (c) instructions for the administration of (a)
14 and (b) separately, sequentially or
15 simultaneously.

16

17

18

1/20

Figure 1A

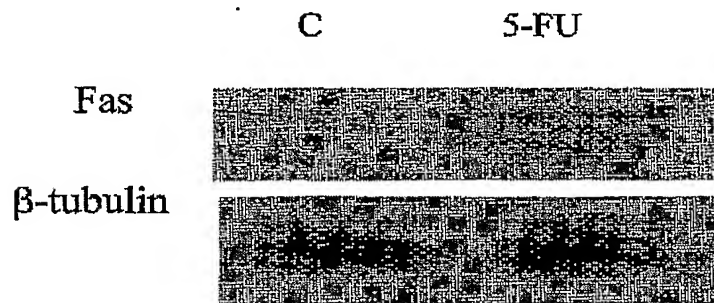
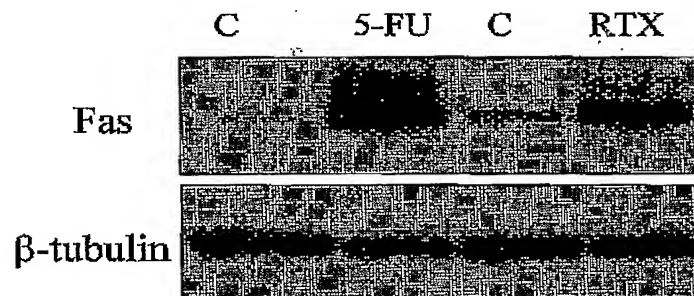
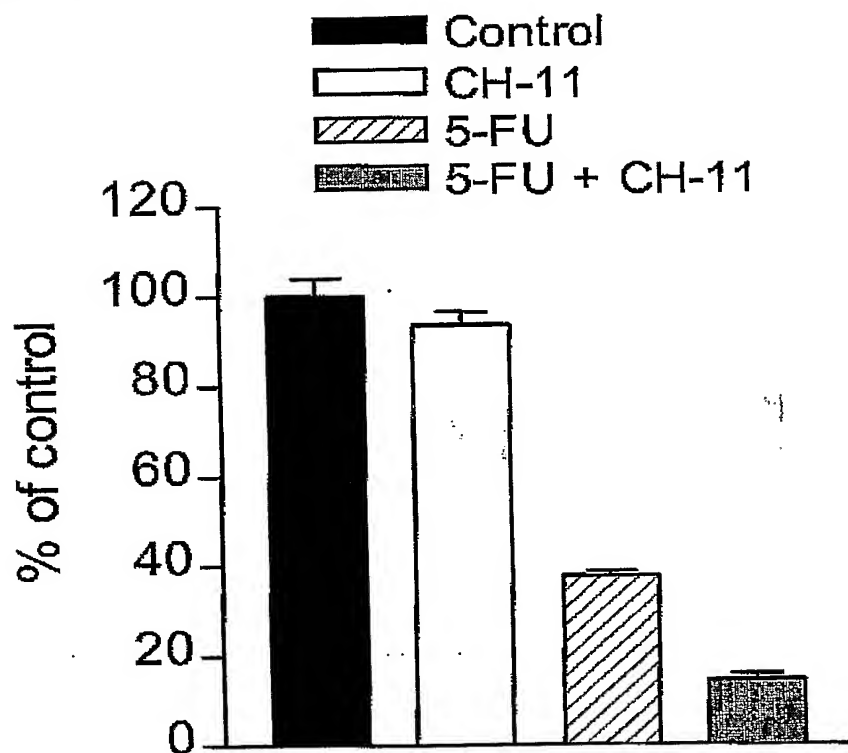


Figure 1B



2/20

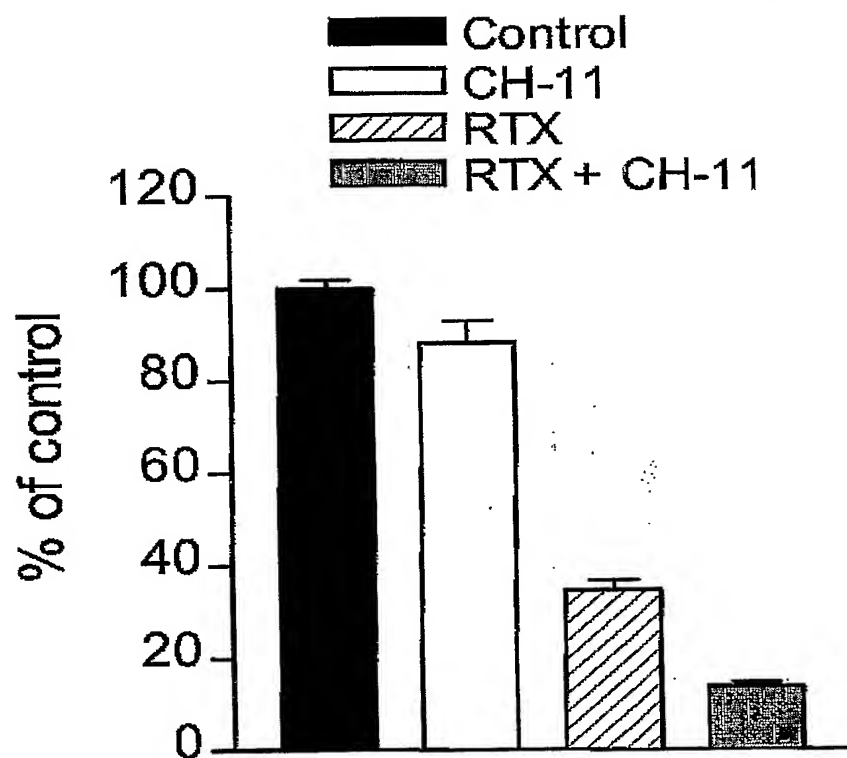
Figure 1C





3/20

Figure 1D





4/20

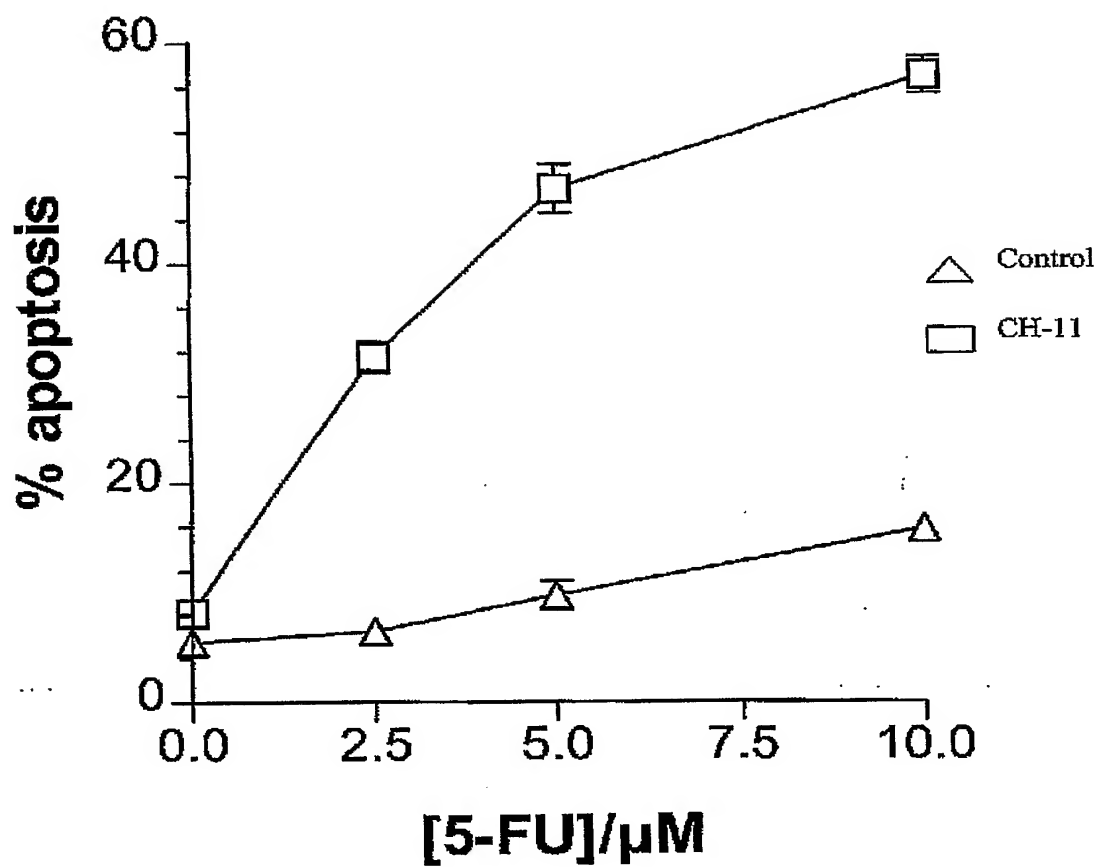


Figure 1E



5/20

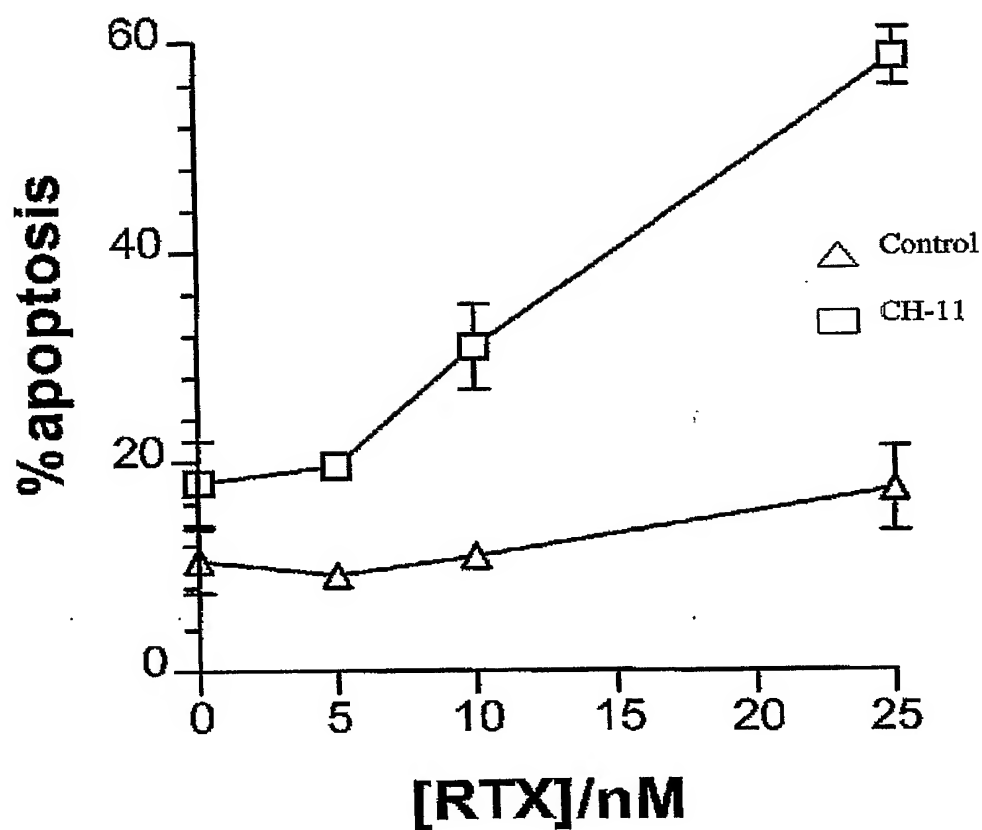


Figure 1F



6/20

Figure 2A

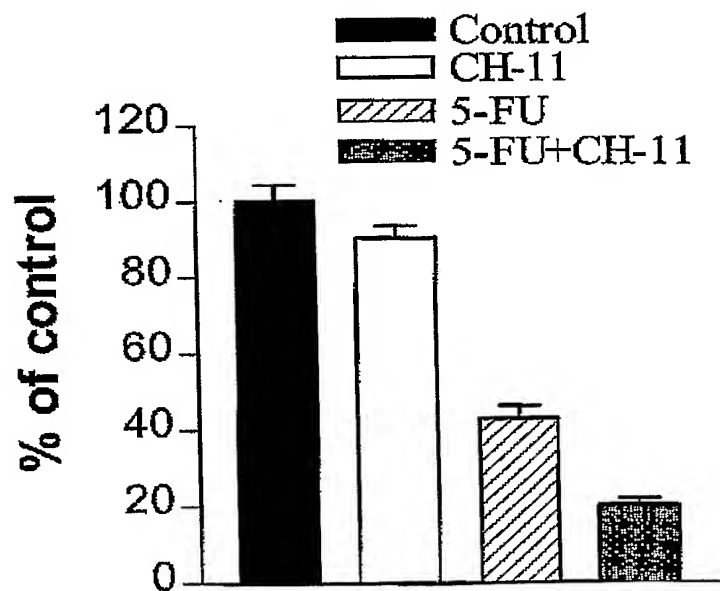
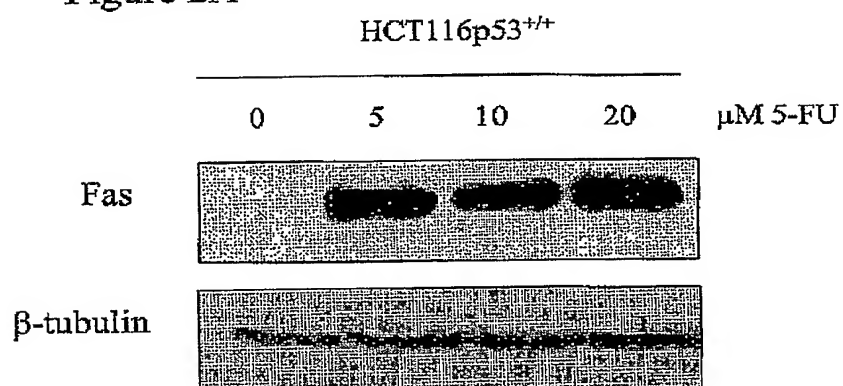


Figure 2B



7/20

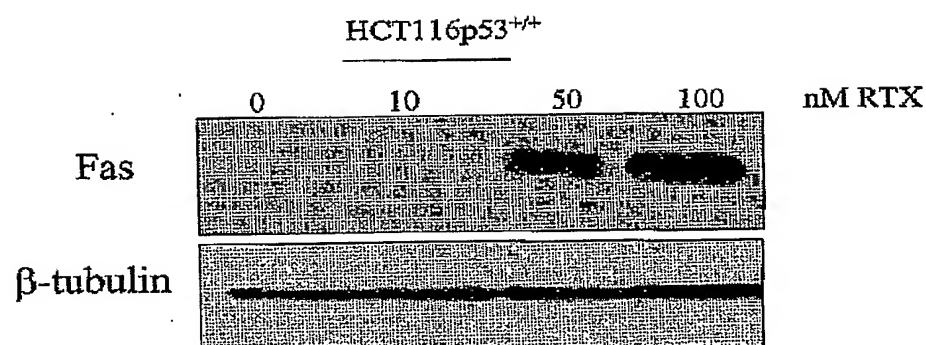


Figure 2C

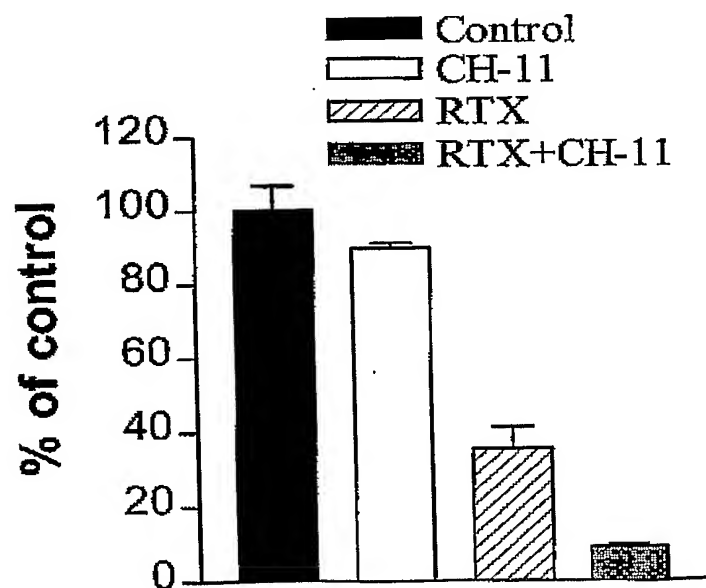


Figure 2D



8/20

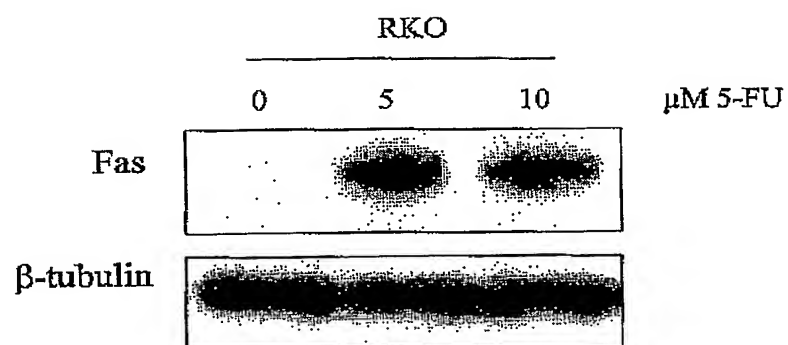


Figure 2E

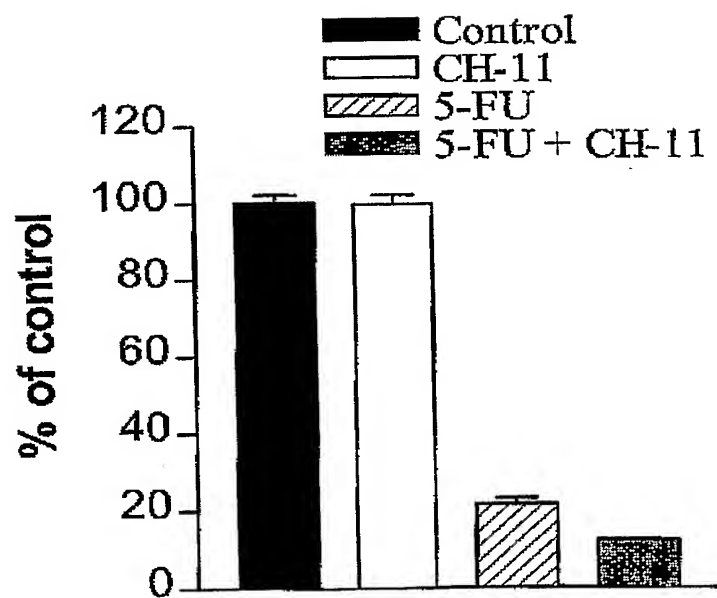


Figure 2F

9/20

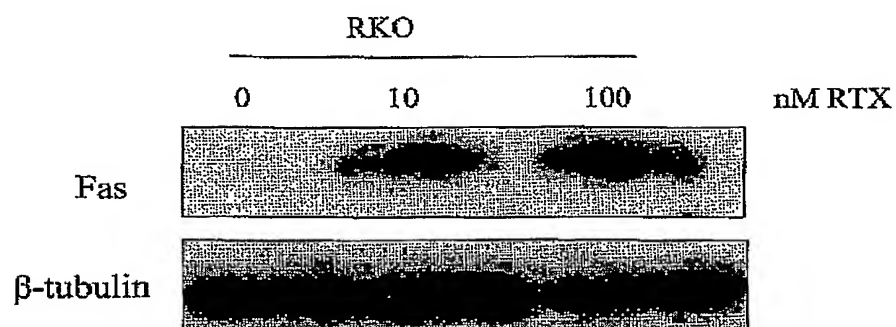


Figure 2G

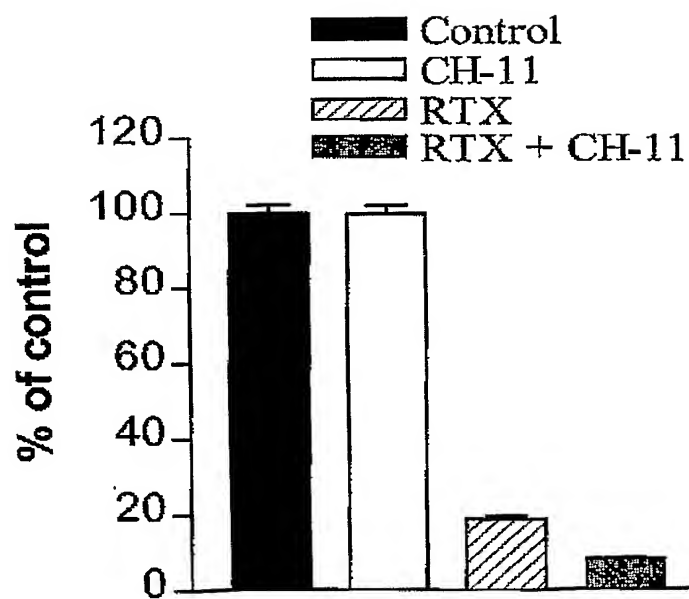


Figure 2H

10/20

Figure 3A

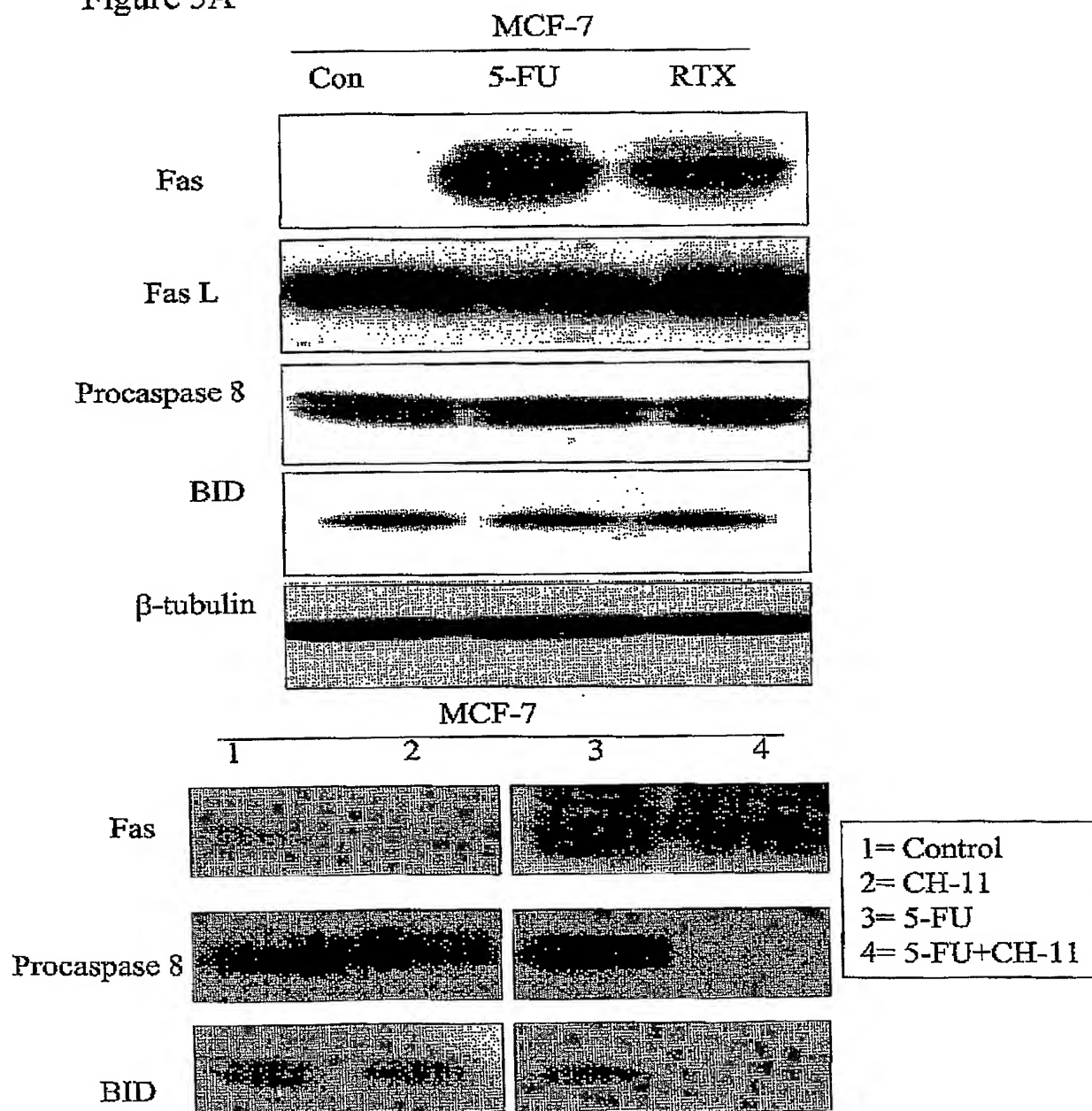


Figure 3B



11/20

Figure 3C

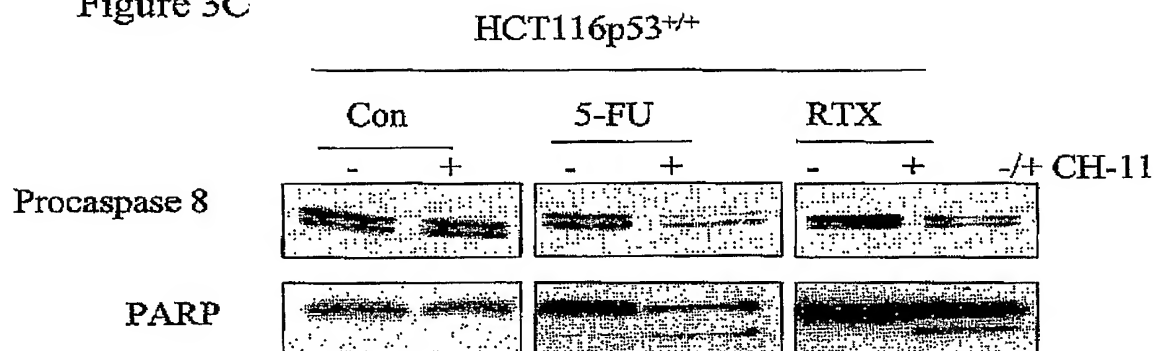


Figure 3D

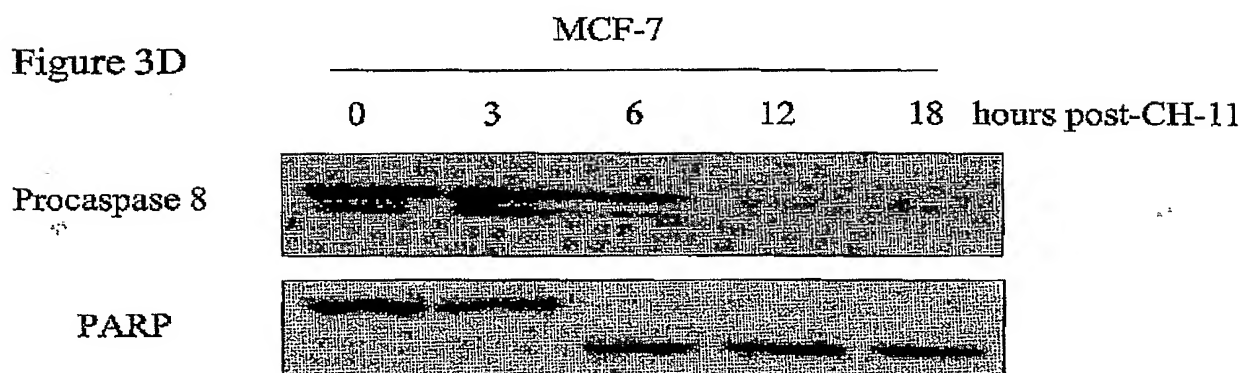
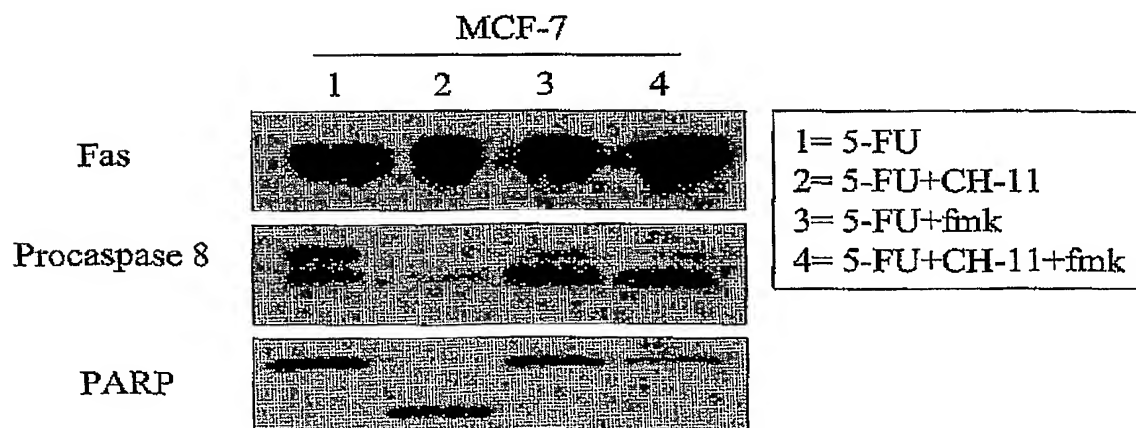


Figure 3E



12/20

Figure 4A

M7TS90

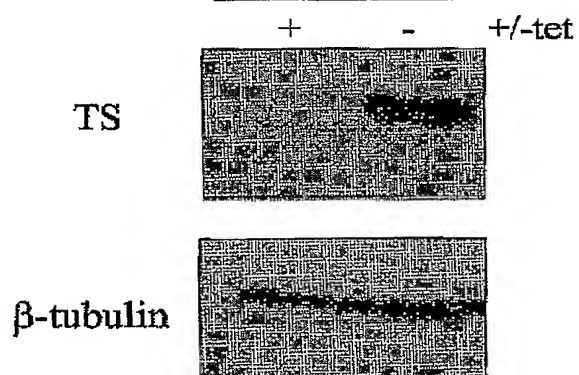
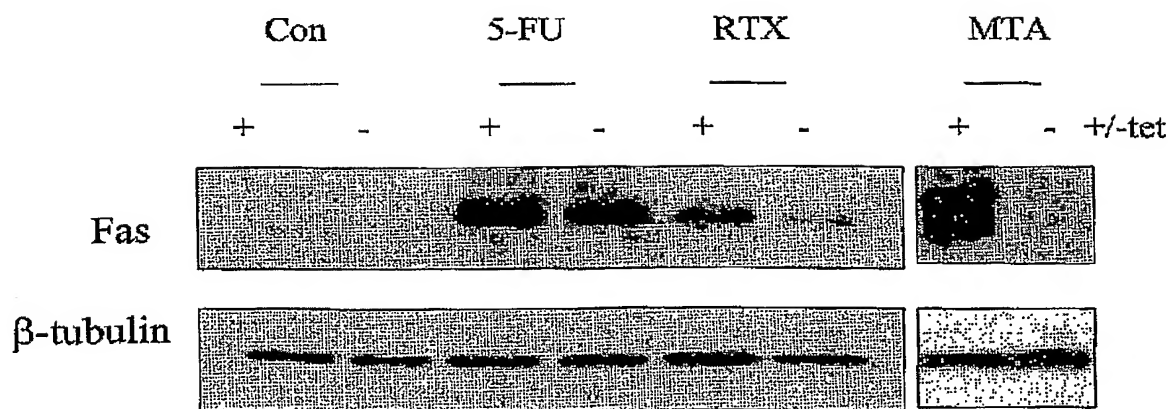


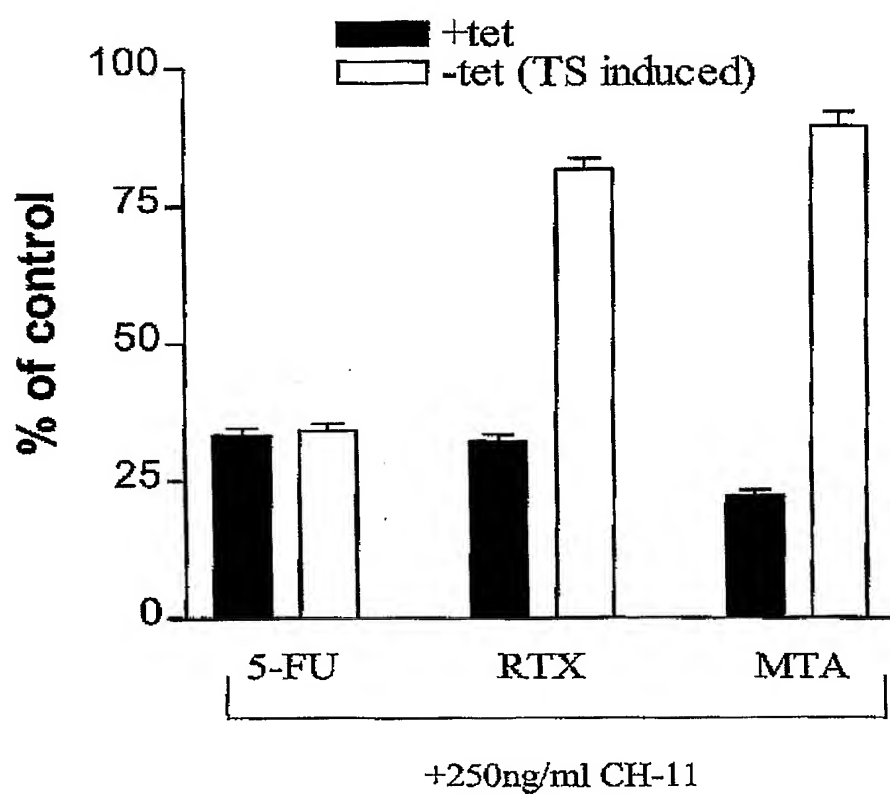
Figure 4B





13/20

Figure 4C





14/20

Figure 4D

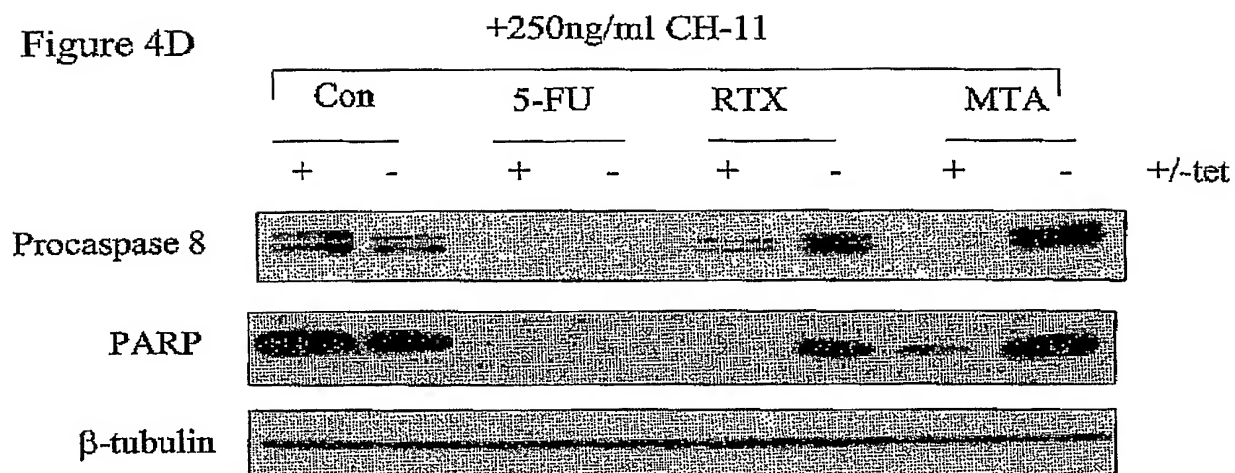
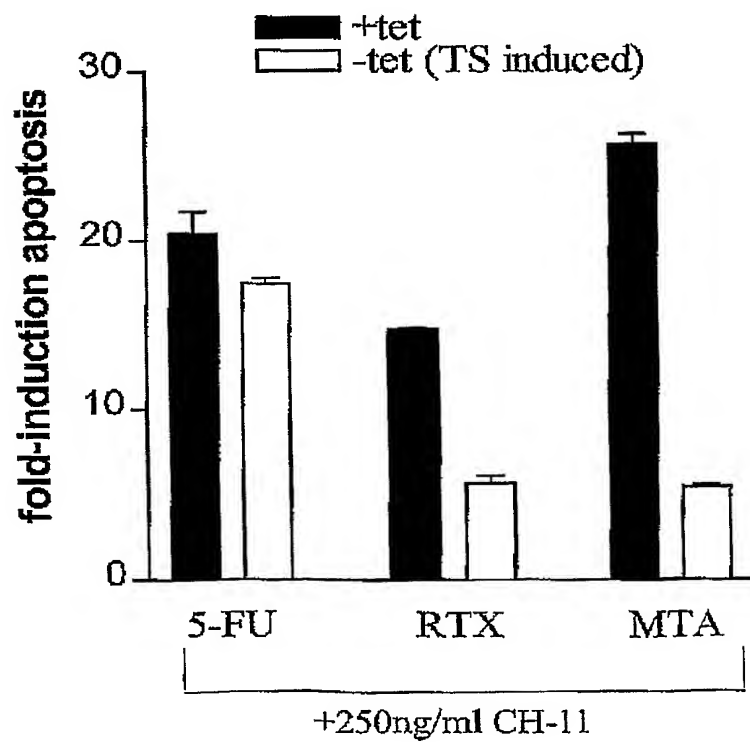


Figure 4E





15/20

Figure 5A

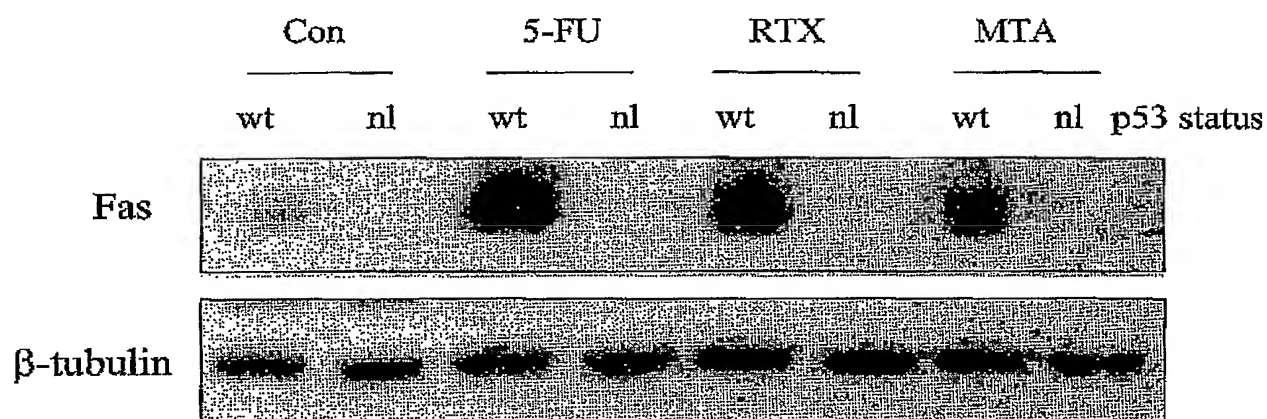


Figure 5B

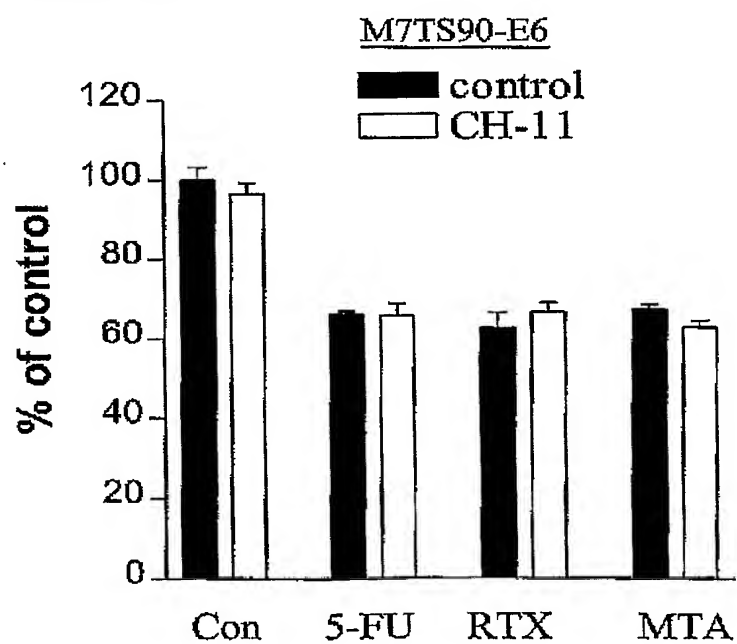


Figure 5C

16/20

+250ng/ml CH-11

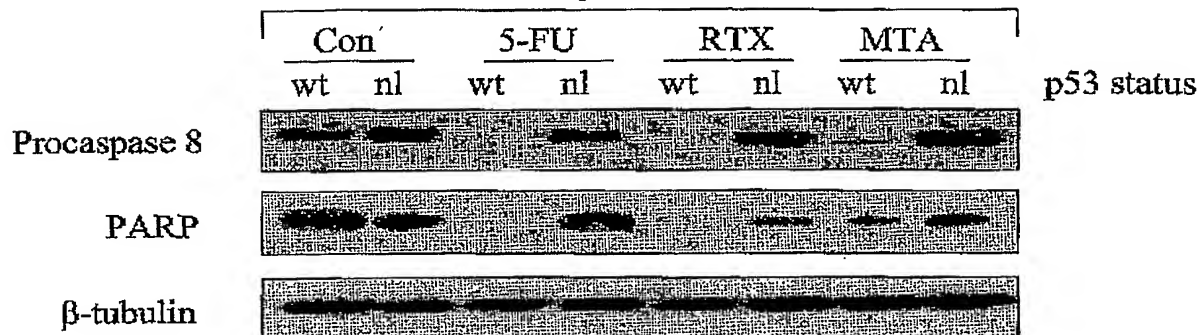
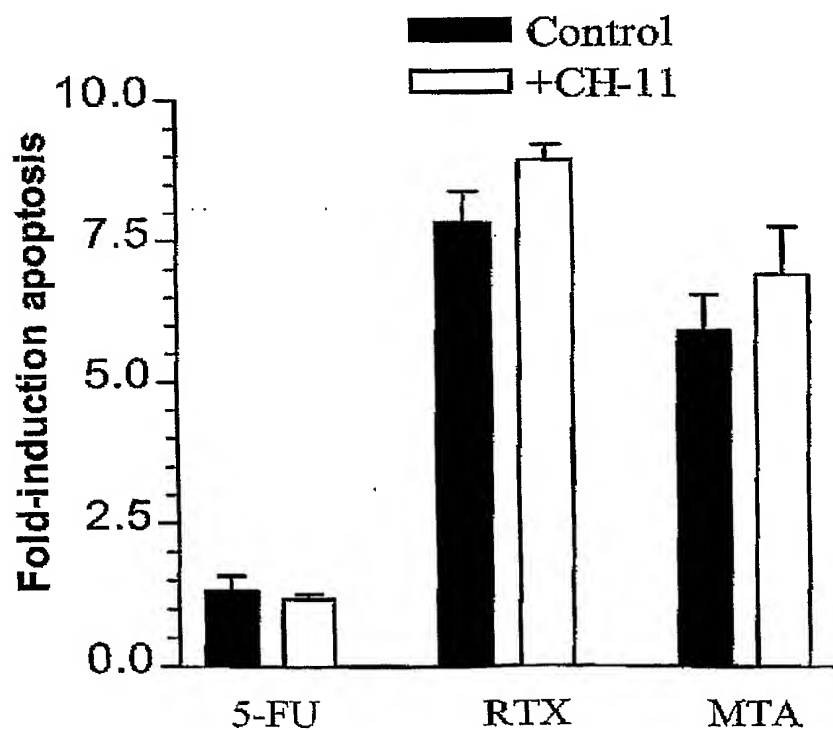


Figure 5D

M7TS90-E6



17/20

Figure 6A

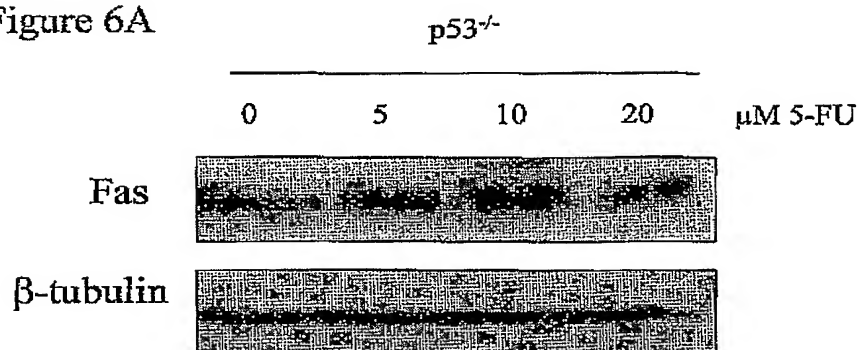
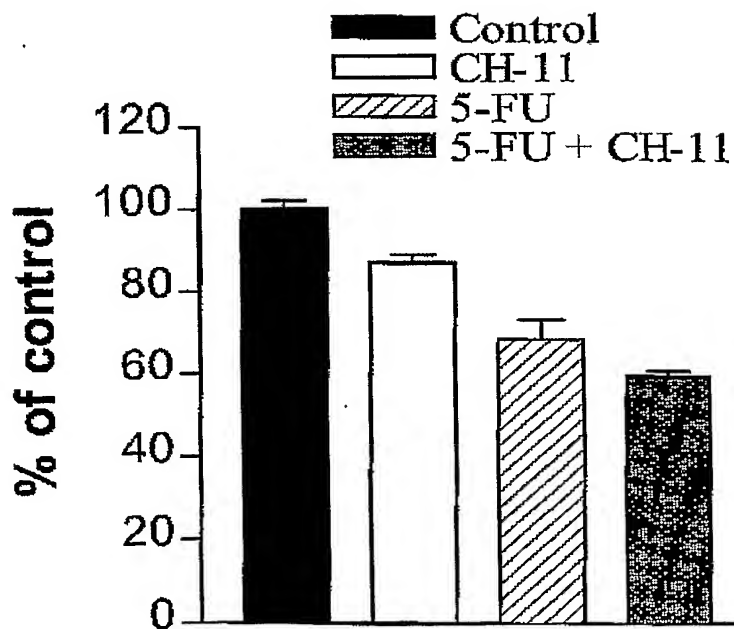


Figure 6B



18/20

Figure 6C

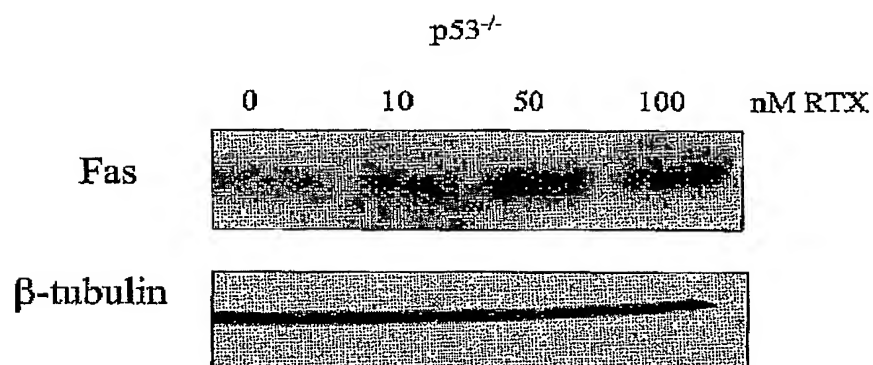


Figure 6D

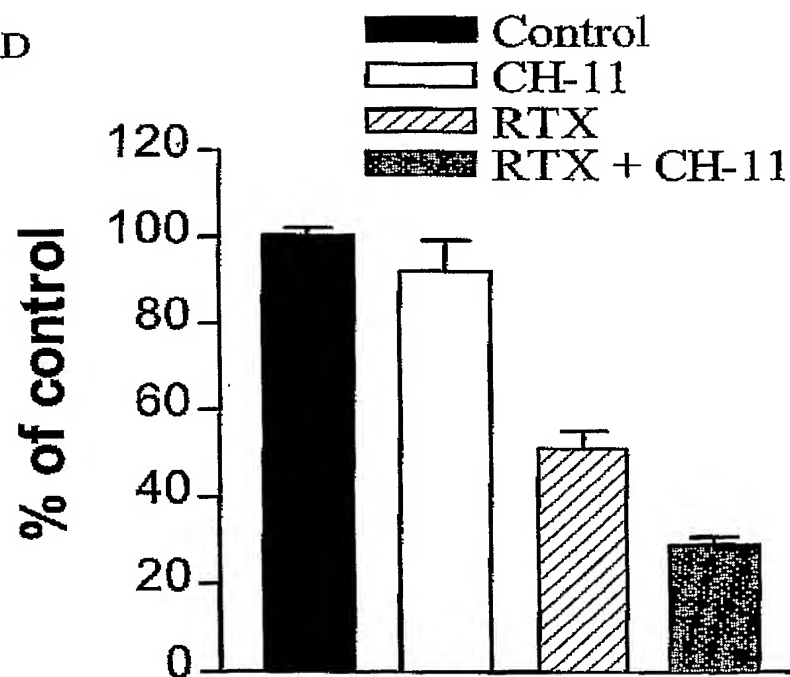




Figure 6E

19/20

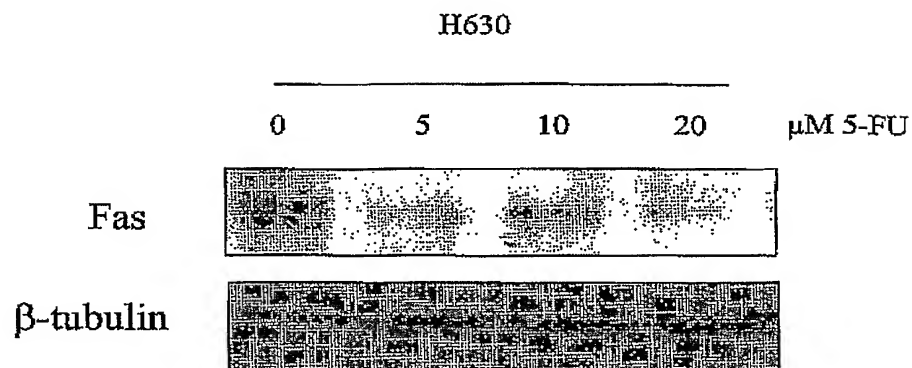
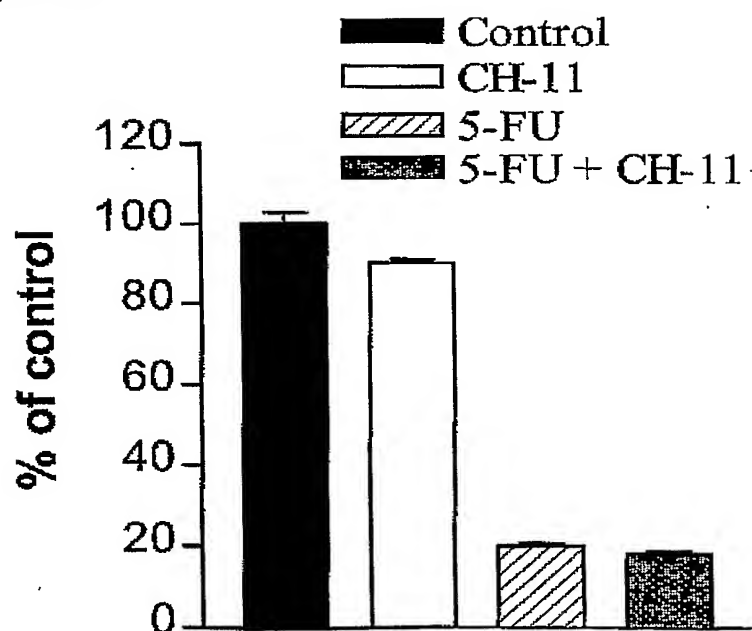
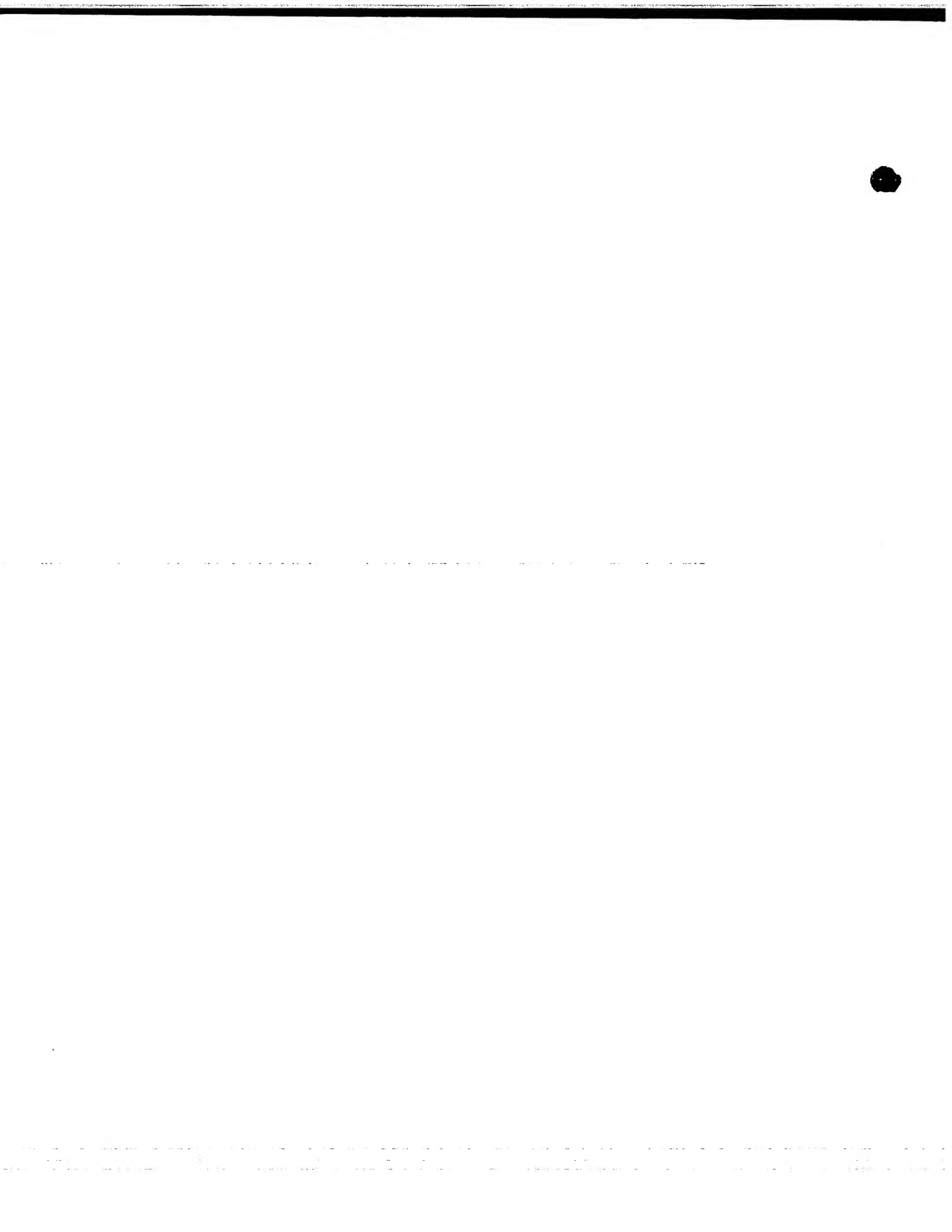


Figure 6F





20/20

Figure 6G

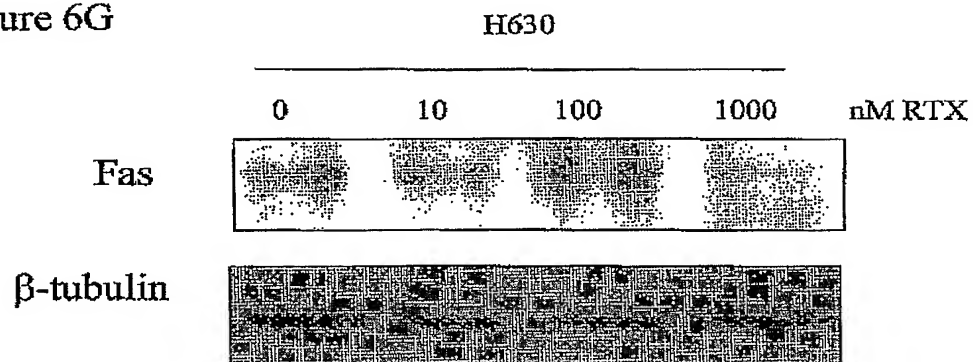
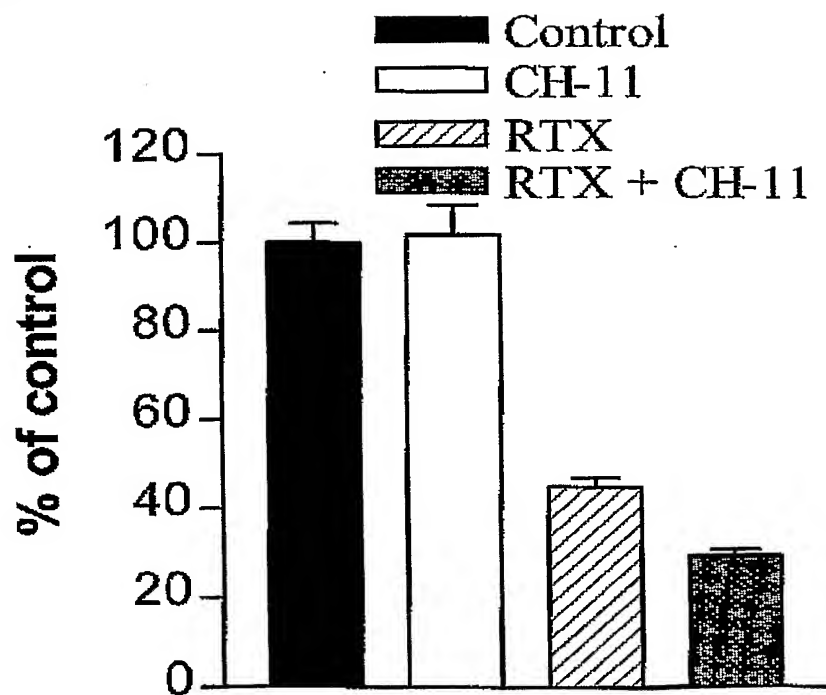


Figure 6H



PCT/GB2004/005006

